Docket No.: PHRM0002-105/PC30125A
PATENT

Appl. Number: 10/523,893
Filed: September 6, 2005

REMARKS

Status of the Claims

Claims 15, 21, 28–30, 51–54, and 57-60 are pending in the application.

Claims 15, 21, 28–30, 51–54, and 57-60 were rejected and the rejection was made final.

Applicants have amended claims 21, 30, 51-54, and 57-60.

Upon entry of this amendment, claims 15, 21, 28–30, 51–54, and 57-60 will be pending.

Summary of the Amendment

Applicants request that claims 21, 30, 51-54, and 57-60 be amended to correct confusing language in reference to the DmGPCR7 protein. Each amended claim included a reference to protein sequences *Lymnaea* lymnokinin (SEQ ID NO: 180) and DLK-2A (SEQ ID NO:183). Each amended claim recited an obvious typographical error that was inadvertently incorporated into the claims after entry of the previous amendment. Applicants have corrected the typographical errors in order to recite a more precise invention. Correction by the amendment herein does not add new matter or raise any new issues. Correction by the amendment places the claims in better condition for allowance or appeal. Accordingly, entry of the amendment is appropriate.

Claim Rejection under 35 U.S.C. §101

Claims 15, 28–30, 51–54, and 57-60 stand rejected under 35 U.S.C. §101 because it is alleged that they are drawn to an invention with no apparent or disclosed specific and substantial credible utility.

The Office contends that Applicants have failed to establish a practical utility because there is no data showing a relationship between DmGPCR-mediated activity and insect death. Thus, the Office contends, those skilled in the art could not employ the assay of the claimed

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invention without making substantial inventive contributions. (Official Action at page 5) Applicants respectfully disagree.

It is well established that in order to make a proper utility rejection, the Office must establish a *prima facie* case that the invention lacks a specific and substantial credible utility. Respectfully, the Office has not made such a case in the instant application. The claimed invention has a specific and substantial credible utility under the law. The burden is on the Office to establish a *prima facie* case. The Office has not met its burden.

To properly support a rejection under 35 U.S.C. §101 the Office has the burden to establish that that one skilled in the art would conclude to that the claimed invention **more likely than not** requires additional inventions in order to have a utility in currently available form. There is no evidence of record to support a finding that one skilled in the art would conclude to that the claimed invention **more likely than not** requires additional inventions in order to have a utility in currently available form. The Office has not met their burden.

Despite the failure of the Office to meet their evidentiary burden with respect to the *prima facie* case that the invention lacks a specific and substantial credible utility, Applicants submit and attach herewith copies of three journal articles that explicitly disclose a relationship between DmGPCR function and insecticides.

Holmes, *et. al.*, *Insect Molecular Biology*, 2000, pages 457-465 (hereinafter "Holmes") discloses a need for an "effective control strategy" for outbreaks of population of the cattle tick as the species has developed a resistance to pesticides (page 457, column 2, paragraphs 1 and 2). Holmes clearly states that:

> The leucokinins are a family of neuropeptides that have been found in several arthropod and invertebrate groups. They have myotropic and diurectic activity in insects. Leucokinins stimulate hindgut contractions in the cockroach, cricket, and locust and increase the rate of secretion in Malpighian tubules. Leucokinins may also serve as neuromodulators of the central nervous system (CNS). ***Thus, because of their multi-functional activities, leucokinin receptors may represent an excellent target for the development of acardial and insecticidal mimetics.***

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(page 457, column 2, paragraph 3, through page 458, column 1, paragraph 1) (emphasis added, references omitted). Holmes discusses the *Drosophila* gene product CG10626 in the context of "presented structural evidence that it most likely encodes a leucokinin-like peptide receptor" (page 459, column 2, paragraph 4). The authors conclude that that Drosophila Malpighian tubule provides a "model system for the analysis of neurohormonal control in insects" (page 461, column 1, paragraph 1). Not only do authors of Holmes establish a connection between the GPCR superfamily and development of acaricides, but authors specifically establish a connection between DmGPCR function and insecticide development.

Radford, *et. al., Journal of Biological Chemistry*, Vol. 277, No. 41, October 11, page 38810-17, 2002 (hereinafter "Radford"), discloses the characterization of the *Drosophila melanogaster* GPCR and leucokinin receptor, CG10626. Radford specifically states:

> The production of primary urine in the Malpighian tubules of insects is driven by the action of diuretic factors. ***The ability of these hormones and their receptors to regulate fluid and ion secretion make them attractive targets for novel insect control approaches. The insect myokinins, known as leucokinins, are one such group of neuropeptides.***

(page 38810, column 1, paragraph 2).

Terhzaz, *et. al., Journal of Experimental Biology*, Vol. 202, pages 3667-3676, 1999, (hereinafter "Terhzaz") discloses the isolation and characterization of a leucokinin-like peptide of *Drosophila melanogaster*. Terhzaz discloses that:

> the best *D. melanogaster* model system for the analysis of neurohormonal control is provided by the renal, or Malpighian, tubules, which regulate salt and water balance by transepithelial secretion.

(page 3667, column 1, paragraph 3). Terhzaz concludes that functions of other individual neuropeptides can be provided using the techniques disclosed.

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Applicants maintain that the disclosure and the invention satisfy the specificity of the utility requirement. It is well settled that the "specific utility" is *specific* to the subject matter claimed and if the subject matter can "provide a well-defined and particular benefit to the public." *In re Fisher*, 421 F.3d 1365, 1371, 76 USPQ2d 1225, 1230 (Fed. Cir. 2005). As discussed in the reply and amendment submitted to the Office on April 4, 2008, Applicants have specifically disclosed the invention's utility for a method of binding an isolated or recombinant DmGPCR with a leucokinin binding partner.

Applicants have identified a specific purpose in performing the functional analysis between a DmGPCR and its leucokinin binding partner as is described in Terhzaz. Terhzaz discloses the functional relationship between DmGPCR and leucokinins in the specific biological activity of insect Malpighian tubules. Study of the method of binding a DmGPCR and leucokinin is reasonably correlative to enhancing and inhibiting GPCR receptor function given the known interaction between DmGPCR7 and leucokinins.

Applicants have identified a specific purpose in identifying modulators of activity between DmGPCR7 and its binding partners. The identification of such a modulator is reasonably correlative to enhancing and inhibiting GPCR receptor function given the known interaction between DmGPCR7 and its binding partners. In fact, such a method was actually used to determine the EC_{50} between DmGPCR7 and its binding partners described on page 108 and 109 of the specification. Therefore, the methods of binding a DmGPCR with a leucokinin present a significant and presently available benefit to the public.

Applicants have disclosed the interaction of DmGPCR7 and a leucokinin. Each of the rejected claims recites the interaction of DmGMPCR7 and a leucokinin. The functions of leucokinin receptors were known to affect Malpighian tubule function in Drosophila. Accordingly, the specific utility of the invention is clearly provided by the disclosure.

Applicants maintain that the substantial utility of the invention is provided in the specification. To satisfy the substantial utility requirement, an asserted use must show that the claimed invention has a significant and presently available benefit to the public. *In re*

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Fisher, 421 F.3d 1365, 1371, 76 USPQ2d at 1230. Any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted as sufficient. MPEP §2107.01(I)(B). Applicants have identified that DmGPCRs in Drosophila may be inhibited in the course of developing a insecticide (See, *e.g.*, Specification page 4). Such an activity is reasonable in light of the specification and is a public benefit. Therefore, the identification of modulators of these compounds present a significant and presently available benefit to the public.

Applicants maintain that a specific and substantial credible utility of the invention is provided in the specification. Applicants have identified that DmGPCRs in Drosophila may be inhibited in the course of developing a insecticide (See, *e.g.*, Specification page 4). Such an activity is reasonable in view of the discussion of Holmes, Radford, and Terhzaz. The evidence of credible utility would lead a person of ordinary skill in the art to conclude that the asserted utility of the invention for developing pesticides is more likely than not true.

Claims 15, 21, 28–30, 51–54, and 57-60 have a specific and substantial credible utility. Applicants respectfully request that the rejection of claims 15, 28–30, 51–54, and 57-60 under 35 U.S.C. §101 be withdrawn.

Claim Rejections Under 35 U.S.C. § 112, first paragraph

Claims 15, 28–30, 51–54, and 57-60 stand rejected under 35 U.S.C. §112, first paragraph, as allegedly failing to adequately teach how to use the instant invention. This rejection has been issued in combination with the rejection issued under 35 U.S.C. §101. Applicants traverse the rejection and respectfully request that the rejection be withdrawn.

Applicants note the rejection under 35 U.S.C. 112, first paragraph, set forth in paragraph 9 of the Official Action is based on grounds related to the "lack of utility" under 35 U.S.C. 101. In particular, it is asserted that the "how to use" requirement is not met for the reasons given with regard to the rejection under 35 U.S.C. 101.

As discussed above, the rejection of claims 15, 28–30, 51–54, and 57-60 was improperly issued under 35 U.S.C. § 101 for allegedly not containing specific and substantial credible utility. Applicants respectfully urge that the application is in compliance with the utility requirement and therefore the rejection under both 35 U.S.C. § 101 and 35 U.S.C. 112, first paragraph, should be withdrawn. As set forth above, the application has a specific and substantial credible utility. Accordingly, the rejection under 35 U.S.C. 112, first paragraph, as well the rejection under 35 U.S.C. §101 rejection should be withdrawn.

Claim Rejections Under 35 U.S.C. § 112, second paragraph

Claims 15, 28-30, and 51-54 stand rejected under 35 U.S.C. §112, second paragraph as being indefinite for failing to particularly point out and distinctly claims the subject matter which the applicant regards as the invention. Applicants submitted an amendment to the Office on April 4, 2008, which addressed this issue. In the Advisory Action dated May 1, 2008, the office stated that:

> the proposed amendments to the claims will avoid the rejection
> in section 7 of the office action mailed 02 February 2008.

(Advisory Action, page 3). The application is in compliance with the requirements of the first and second paragraphs of section 112. Applicants respectfully request that the rejections of claims 15, 28-30, 51-54 and 57-60 under 35 U.S.C. § 112, first paragraph, and 35 U.S.C § 112, second paragraph, be withdrawn.

Claims 21, 51-53 and 57-60 stand rejected under 35 U.S.C § 112, second paragraph, allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter of the claimed invention. Claim 21 has been deemed vague due to a failure to update its dependency when the limitations of claim 20 were incorporated into claim 15. Claims 51-53 and 57-60 have been deemed vague and indefinite because Markush language rendered unnecessary was not deleted in the earlier amendment. The amendment submitted on April 4, 2008, and entered by the Office obviates the basis of the rejection. Nevertheless, the Office

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did not provide any guidance in the Advisory Action as to whether the rejection remains pending.

The application is in compliance with the requirements of the second paragraph of section 112. Applicants respectfully request that the rejection of claim 21, 51-53 and 57-60 under 35 U.S.C. § 112, second paragraph, be withdrawn.

Conclusion

Upon entry of this amendment, claims 15, 21, 28–30, 51–54, and 57-60 will be in condition for allowance. Applicants respectfully request that the amendment be entered and that the claims be allowed at this time. An early Notice of Allowance is therefore earnestly solicited. Applicants invite the Examiner to contact the undersigned attorney at 610.640.7855 to clarify any unresolved issues raised by this response.

As indicated on the transmittal accompanying this response, the Commissioner is hereby authorized to charge any debit or credit any overpayment to Deposit Account No. 50-0436.

Respectfully submitted,

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Cloning and transcriptional expression of a leucokinin-like peptide receptor from the Southern cattle tick, *Boophilus microplus* (Acari: Ixodidae)

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Abstract

Leucokinins are invertebrate neuropeptides that exhibit myotropic and diuretic activity. Only one leucokinin-like peptide receptor is known, the lymnokinin receptor from the mollusc *Lymnaea stagnalis*. A cDNA encoding a leucokinin-like peptide receptor was cloned from the Southern cattle tick, *Boophilus microplus*, a pest of cattle world-wide. This is the first neuropeptide receptor known from the Acari and the second known in the subfamily of leucokinin-like peptide G-protein-coupled receptors. The deduced amino acid sequence exhibits 40% identity to the lymnokinin receptor. The receptor transcript is present in all tick life stages as determined by semiquantitative reverse transcription polymerase chain reaction. We also propose that the sequence AAF50775.1 from the *Drosophila melanogaster* genome (CG10626) encodes the first identified insect leucokinin receptor.

Keywords: neuropeptide receptor, Acari, G-protein-coupled receptor, developmental expression, cattle fever tick, *Drosophila melanogaster*.

Introduction

The Southern cattle tick or cattle fever tick, *Boophilus microplus*, is the most important tick pest of cattle in tropical and subtropical regions of the world because of its ability to transmit *Babesia* spp., the haemoparasites that cause cattle fever (Nuñez *et al.*, 1985). Ticks cause greater economic

losses in livestock production world-wide than any other group of external parasites (Bowman *et al.*, 1996). The compounded economic impact of cattle fever and tick parasitism prior to their eradication from the Southern USA in 1960 was estimated at over one billion dollars annually (Graham & Hourrigan, 1977). However, the ongoing detection of sporadic outbreaks of this tick in Southern Texas is evidence that reintroduction of this pest to the USA presents a serious threat (Anonymous, 2000). Control of *B. microplus* has become increasingly difficult world-wide because it has rapidly developed resistance to pesticides. In Australia, where strains of the tick are resistant to all commonly used acaricides (Baxter & Barker, 1999), the loss to the cattle industry is one hundred million dollars annually (Angus, 1996). In Mexico, resistance to organophosphates (Rosario-Cruz *et al.*, 1997) and pyrethroids (He *et al.*, 1999) has been reported.

One effective control strategy for the Southern cattle tick may be endocrine disruption. Mimetics of ecdysone and juvenile hormone, such as tebufenozide and methoprene, respectively, are effective insecticides (Wing *et al.*, 1988; Jones, 1995). However, a third group of currently unexploited hormones, the peptide hormones, may have a great potential for control because they are master regulators and affect a number of physiological processes (Keeley & Hayes, 1987). In order to develop neuropeptide mimetics useful in pest control, the chemical and conformational requirements of neuropeptide–receptor interactions must be understood (Nachman *et al.*, 1993). Although several neuropeptides have been isolated from insects (Nässel, 1996), very few of their receptors have been identified.

The leucokinins are a family of neuropeptides that have been found in several arthropod and invertebrate groups (Nässel, 1996). They have myotropic and diuretic activity in insects. Leucokinins stimulate hindgut contractions in the cockroach, cricket, and locust (Holman *et al.*, 1987; Holman *et al.*, 1990; Schoofs *et al.*, 1992) and increase the rate of secretion in Malpighian tubules (Pannabecker *et al.*, 1993; Veenstra *et al.*, 1997; O'Donnell *et al.*, 1998; Cady & Hagedorn, 1999a; Holman *et al.*, 1999). Leucokinins may also serve as neuromodulators of the central nervous system (CNS) (Nässel, 1996). Thus, because of their multifunctional activities, leucokinin receptors may represent

an excellent target for the development of acaricidal and insecticidal mimetics. There is only one report on the biochemical characterization of a 54 kDa leucokinin receptor from an insect (Pietrantonio et al., 2000). The leucokinin-like receptor subfamily is to date comprised of only one receptor, the lymnokinin receptor, cloned from a mollusc, the pond snail Lymnaea stagnalis (Cox et al., 1997).

We have used polymerase chain reaction (PCR) and molecular techniques to obtain a cDNA of a novel leucokinin-like peptide receptor from the Southern cattle tick, B. microplus. Here we report the characterization of this cDNA and show the developmental expression of this receptor by reverse transcription (RT)-PCR experiments. This work constitutes the first evidence of leucokinin-like regulated signal transduction in the Acari. The leucokinin-like peptide receptor is the first neuropeptide receptor to be cloned from the Acari; the second G-protein-coupled receptor (GPCR) known from

B. microplus and the second member of the leucokinin-like receptor subfamily. In addition, the sequence similarity of the Boophilus receptor to the gene CG10626 in the recently published Drosophila melanogaster genome may indicate that CG10626 encodes the first identified insect leucokinin receptor (Adams et al., 2000).

Results

The PCR with degenerate primers corresponding to trans-membrane (TM) regions III and VI, highly conserved in many GPCRs (Cox et al., 1997), amplified DNA products of about 450 bp and 600 bp. Comparisons to the GenBank database showed that the amplified sequence of 450 bp was most similar to those of the kinin receptor family. Nested gene-specific primers were then designed on the basis of the nucleotide sequence of the 450 bp PCR product and used

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                                                                       CGCCCGG    7
GCAGGTCGCTAAGCGCGGTCGGCAGCATTCGCGCGCCGCTCGGCAATCCGCGCGCACCACGAACGGCCGTTCCGCTATGGTGCCACA   94

ATG ACC TCG CTG CCC GGC ATG ACC CTC GAC CCG TCG GCT CCG CCA CCC CTG CTG CTG GAC AGC TCG   160
 M   T   S   L   P   G   M   T   L   D   P   S   A   P   P   P   L   L   L   D   S   S      22

TAC GTG TCA CCG GAC TAC GGG AAC CTG TCG CTG CTG TCA TCG TTG CCG GCA GCA AAC ATC AGT TCC   226
 Y   V   S   P   D   Y   G   N   L   S   L   L   S   S   L   P   A   A   N   I   S   S      44

AAC AAG TTG TAC CAG GTT CCC GTC GGC TTC ATC GTG CTC CTC TCC ATA TTC TAC GGC ATC ATA TCA   292
 N   K   L   Y   Q   V   P   V   G   F   I   V   L   L   S   I   F   Y   G   I   I   S      66

CTG GTG GCC GTC GCC GGC AAC TTC ATG GTC ATG TGG ATC GTG GCC ACG TCA CGG CGC ATG CAG ACA   358
 L   V   A   V   A   G   N   F   M   V   M   W   I   V   A   T   S   R   R   H   Q   T      88

GTC ACC AAC TTC TTC ATC GCC AAT CTG GCC GTA GCC GAC ATC ATC ATC GGA CTG TTC TCC ATC CCG   424
 V   T   N   F   F   I   A   N   L   A   V   A   D   I   I   I   G   L   F   S   I   P      110

TTC CAG TTC CAG GCA GCT CTG CTG CAG CGC TGG GTG CTG CCC GAG TTC ATG TGC GCC TTC TGC CCC   490
 F   Q   F   Q   A   A   L   L   Q   R   W   V   L   P   E   F   M   C   A   F   C   P      132

TTC GTA CAG GTG CTC TCG GTC AAC GTG TCC ATA TTC ACG CTG ACC GCC ATC GCA CTG GAC CGC TAC   556
 F   V   Q   V   L   S   V   N   V   S   I   F   T   L   T   A   I   A   L   D   R   Y      154

CGC GCC GTC ATG TCG CCC CTT AAG GCT CGC ACC ACC AAG CTG CGC GCA AAG TTC ATC ATC TGC GGT   622
 R   A   V   M   S   P   L   K   A   R   T   T   K   L   R   A   K   F   I   I   C   G      176

ATC TGG ACG CTC GCG GTT GCG GCC GCT CTG CCG TGC GCA CTC GCT CTC CGT GTC GAG ACG CAG GTC   688
 I   W   T   L   A   V   A   A   A   L   P   C   A   L   A   L   R   V   E   T   Q   V      198

GAG TCT CAC GCG CTC AAC CTG ACT AAG CCG TTC TGC CAC GAG GTT GGC ATT TCG CGC AAG GCC TGG   754
 E   S   H   A   L   N   L   T   K   P   F   C   H   E   V   G   I   S   R   K   A   W      220

CGC ATC TAT AAC CAC GTC CTC GTG TGT CTG CAG TAC TTT TTC CCG CTG CTC ACC ATC TGT TTC GTG   820
 R   I   Y   N   H   V   L   V   C   L   Q   Y   F   F   P   L   L   T   I   C   F   V      242

TAC GCG CGC ATG GGC CTC AAG CTC AAG GAG AGC AAG TCT CCC GGC AAT GCC CAG GGT GCG CGC GAC   886
 Y   A   R   M   G   L   K   L   K   E   S   K   S   P   G   N   A   Q   G   A   R   D      264

GCC GGC ATC CTC AAG AAC AAA AAG AAG GTG ATC AAG ATG CTG TTT GTC ATC GTG GCA CTG TTC GCC   952
 A   G   I   L   K   N   K   K   K   V   I   K   M   L   F   V   I   V   A   L   F   A      286

TTT TGC TGG CTC CCT TAT CAG TTA TAC AAC ATT CTC CGG GAA GTT TTC CCA AAG ATT GAC AAG TAC   1018
 F   C   W   L   P   Y   Q   L   Y   N   I   L   R   E   V   F   P   K   I   D   K   Y      308

AAG TAC ATA AAC ATA ATC TGG TTC TGT ACA CAC TGG CTG GCC ATG AGT AAC TCC TGC TAC AAT CCT   1084
 K   Y   I   N   I   I   W   F   C   T   H   W   L   A   M   S   N   S   C   Y   N   P      330

TTC ATT TAT GCC ATC TAC AAC GAG CGC TTC AAG CGC GAG TTC GCC ACT CGC TGT ACT TGC GGC GGT   1150
 F   I   Y   A   I   Y   N   E   R   F   K   R   E   F   A   T   R   C   T   C   G   G      352

CAT CGC TAC AAG TCC CCC AAR AGC CGC TTC GCC TCG TAC GAG CAG GAA GAC AAC TCG ACC ATC ATT   1216
 H   R   Y   K   S   P   K   S   R   F   A   S   Y   E   Q   E   D   N   S   T   I   I      374

GTG TCT ATG CGG CAC TCA TTC CGG CTG AGC TTC AAG AAT TCC GCG CCG CTC AAA GCG AGC ACG CAG   1282
 V   S   M   R   H   S   F   R   L   S   F   K   N   S   A   P   L   K   A   S   T   Q      396

GTG TAA TTAGAGTCAACCACCAGTGAAATCCTATCAGTGCACGTGCATCGGGAAGAAGAAACAGGAGACTGWGCGGSGCCCCCTA  1367
 V                                                                                          397

YTTCCTTCGTGGCGCTTTCTYCGCGACGATGGTGATATCTGTGCATGTTGAACCCGATCTGCGCAGCACCCATTTGCAAATGCGCCA 1454
GCCCGGAAGGCGACCACCCGCCATGGCTCAAATGGCCGTGCCCCACCGACCTGCTTCAACACGGAACTTGAGCCCTGAACTTGACCA 1541
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Figure 1. Nucleotide and deduced amino acid sequence of the putative leucokinin-like peptide receptor cDNA from the Southern cattle tick, B. microplus (GenBank/EMBL accession number AF228521). Amino acids corresponding to predicted transmembrane regions have been underlined. Possible glycosylation sites are marked with a diamond.

in 5' and 3' rapid amplification of cDNA ends (RACE) to amplify the full-length receptor cDNA. The primers were designed to encompass the majority of the 450 bp product in the 3' and 5' RACE reactions, respectively, to unequivocally obtain the corresponding cDNA ends. The 3' RACE product was 2 kb and the 5' RACE product was 850 bp. The sequences of the 3' and 5' RACE fragments were identical to each other in the expected region of overlap. The full-length cDNA was 2.7 kb, out of which 1541 bp are shown in Fig. 1. The complete sequence of the cDNA has been deposited in GenBank (AF228521).

The identified open reading frame (ORF) consists of 1194 bp and encodes a protein of 397 residues with a predicted molecular mass of 44.9 kDa (Fig. 1). Two possible start codons, beginning at positions 95 and 123, were identified within an ORF that terminates with a stop codon at position 1286. The first ATG at positions 95–97 was presumed to be the actual start codon because neighbouring bases more closely matched the optimal sequence for translation initiation, GCC(A/G)CCATGG (Lewin, 1997).

BLAST P searches of GenBank showed that this sequence encoded a GPCR that was similar to the lymnokinin receptor from pond snail (40.3% identity) (Cox *et al.*, 1997) and other invertebrate neuropeptide receptors (Fig. 2, Table 1). The gene product from the *D. melanogaster* genomic sequence, CG10626, resulted 46.9% identical to the *B. microplus* receptor sequence. These sequences were analysed for similarity with DNASTAR (Table 1, Fig. 2). Kyte and Doolittle hydrophilicity plots of the *B. microplus* receptor, lymnokinin receptor and CG10626 (Fig. 3) reveal seven putative transmembrane regions. In the *B. microplus* receptor, two cysteine residues, Cys 128 and Cys 210, located in the first and second extracellular loops, respectively, are expected to form a disulphide bond. The location of these residues is consistent in almost all GPCRs (Watson & Arkinstall, 1994; Schöneberg *et al.*, 1999). The N-terminal region contains two asparagine residues, Asp 30 and Asp 41, within a glycosylation consensus sequence N-X-S/T (Lewin, 1997). There is also a third glycosylation site, Asp 204, in the predicted second extracellular loop. Two cysteine residues in the intracellular C terminus (Cys 348, Cys 350) represent likely sites for palmitoylation (Iismaa *et al.*, 1995).

The receptor sequence used for semiquantitative RT-PCR experiments and synthesis of the probe for the Southern blot corresponds to the C terminus, the region known to be the least similar among GPCRs from the same subfamilies and thus often used to identify specific receptors. In order to ensure specificity, the antisense primer was designed within the 3' untranslated region and the sense primer in a region of low similarity corresponding to the third extracellular loop (Figs 1 and 4). Receptor messenger RNA (mRNA) was present in all life stages of the Southern cattle tick as determined by semiquantitative RT-PCR (Fig. 4A). These results suggested that receptor expression was highest in larvae and adult females. In these experiments the amount of amplified receptor cDNA produced by RT-PCR (318 bp, Fig. 4A) had been normalized by comparison with the amplification of *B. microplus* β-actin (330 bp, Fig. 4A). A pixel-density analysis indicated that the maximal amplification of receptor PCR product was in females, followed by larvae, eggs, nymphs and males (Fig. 4A). The amplified products were confirmed to be identical to the cloned receptor by Southern blot (Fig. 4B).

Discussion

The leucokinin-like peptide receptor from the cattle fever tick *B. microplus* is the first neuropeptide receptor cloned from a tick and the second receptor identified in this species. Only four other receptors have been cloned from ticks, an octopamine receptor from *B. microplus* (AJ0107043) (Baxter & Barker, 1999), and three nuclear receptors from *Amblyomma americanum* (Guo *et al.*, 1997; Palmer *et al.*, 1999).

Knowledge of tick endocrinology is limited (Sonenshine, 1991; Lomas *et al.*, 1997). However, by analogy with insects, tissues expressing leucokinin receptors in ticks may be involved in water balance or neuromodulation, such as the Malpighian tubules and hindgut, or the CNS (synganglion), respectively. In insects, leucokinins act through increases in intracellular calcium in an inositol 1,4,5-triphosphate (IP$_3$)-dependent and cyclic nucleotide-independent mechanism to stimulate secretion in isolated Malpighian tubules (O'Donnell *et al.*, 1998; Cady & Hagedorn, 1999a,b; Terhzaz *et al.*, 1999). Receptor binding of lymnokinin results in an increase of intracellular calcium (Cox *et al.*, 1997); it is likely that the *B. microplus* receptor induces intracellular calcium release.

In the arthropods there is little information on neuropeptide hormone receptors, and no leucokinin receptors have been identified. Only four neuropeptide receptors are known from insects, three from *D. melanogaster* and one from the stable fly, *Stomoxys calcitrans* (Li *et al.*, 1991, 1992; Rosay *et al.*, 1995; Monnier *et al.*, 1992; Guerrero, 1997). The identification of leucokinin receptors is key to further understanding of leucokinin hormonal functions. The amino acid sequence of the *B. microplus* receptor is most similar to the CG10626 *Drosophila* gene product (Adams *et al.*, 2000) and to the lymnokinin receptor from *L. stagnalis* (Table 1), which has been characterized as a leucokinin-like peptide receptor and is the first member of a new subfamily of GPCRs (Cox *et al.*, 1997). It is also similar to the neuropeptide Y receptor (NPY) from *Drosophila* (Li *et al.*, 1992). The function of *D. melanogaster* gene product CG10626 (AAF50775) has not been characterized unequivocally. Various functions ranging from cell motility to signal transduction are mentioned <http://flybase.bio.indiana.edu/.bin/fbidq.html?FBgn0035610>, however, we presented structural evidence that it most likely encodes a leucokinin-like peptide receptor (Fig. 2).
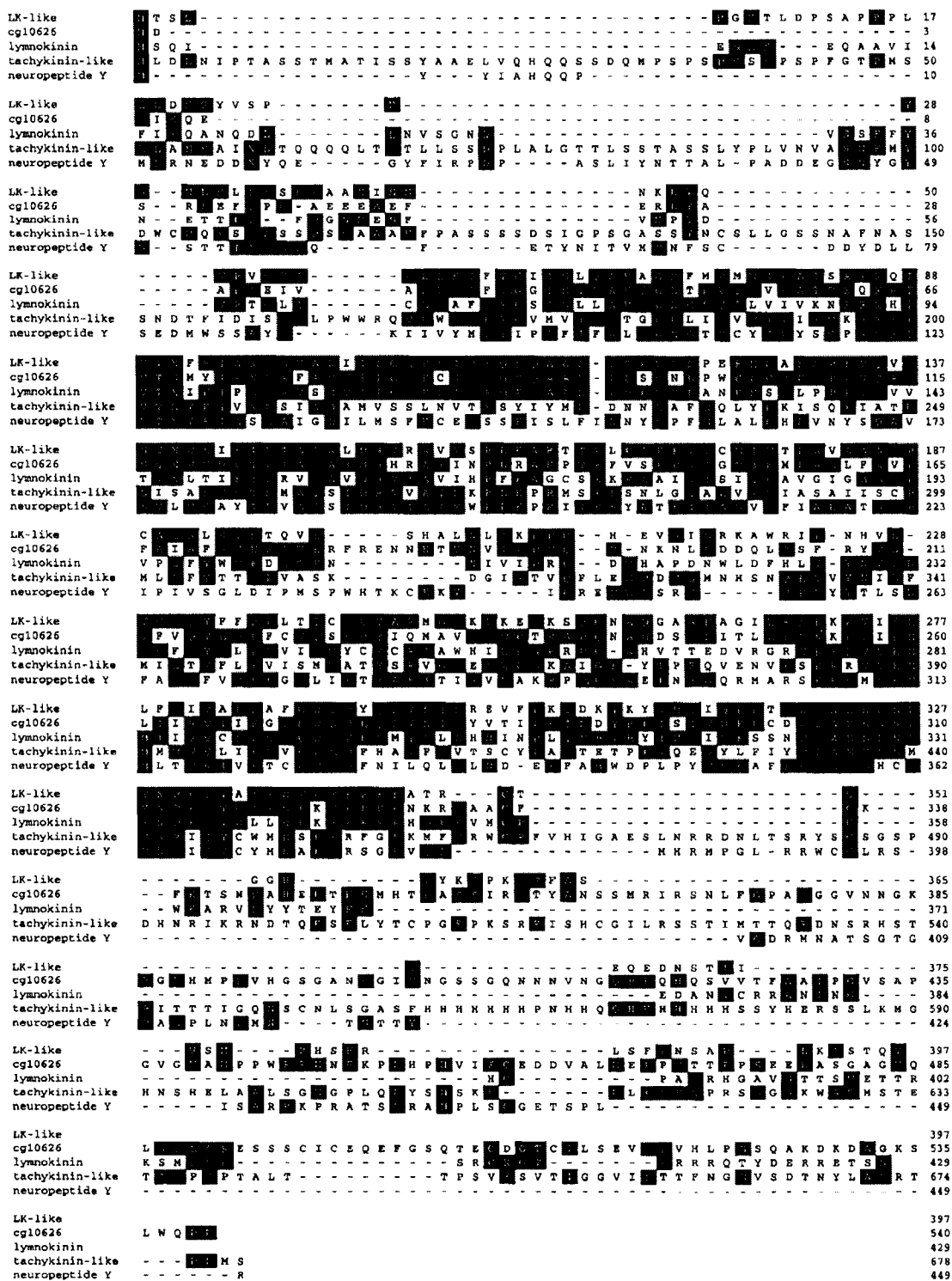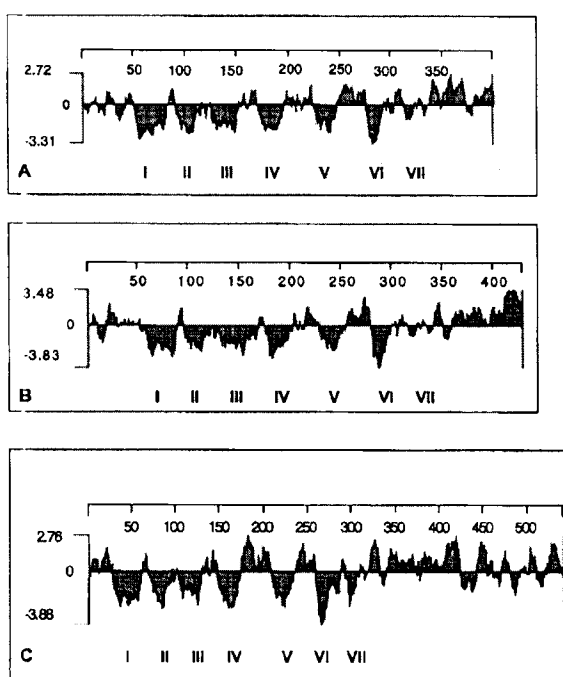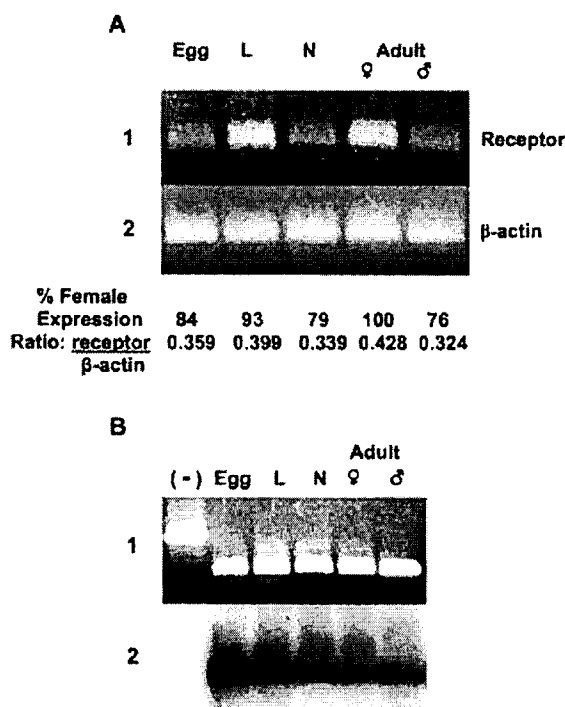
Figure 2. Amino acid alignment of the putative leucokinin-like peptide receptor from the Southern cattle tick, *B. microplus*, with four similar invertebrate neuropeptide receptors. Identical residues are noted as white text on a black background. Aligned sequences are the *D. melanogaster* CG10626 gene product (AE003566), *L. stagnalis* lymnokinin receptor (U84499), *Stomoxys calcitrans* tachykinin-like receptor (U52347), and *D. melanogaster* NPY receptor (M81490).

Table 1. Amino acid identity of the *B. microplus* receptor to other invertebrate neuropeptide receptors and probability values from BLAST searches

Receptor	Identity	BLAST P probability
CG10626 *Drosophila melanogaster* (fruit fly)	46.9%	1e-100
Lymnokinin *Lymnaea stagnalis* (pond snail)	40.3%	2e-79
Tachykinin-like *Stomoxys calcitrans* (stable fly)	29.0%	1e-50
Neuropeptide Y *Drosophila melanogaster*	28.0%	2e-49

	Egg	L	N	Adult ♀	♂	
% Female Expression	84	93	79	100	76	
Ratio: receptor / β-actin	0.359	0.399	0.339	0.428	0.324	

Figure 4. (A) Semiquantitative RT-PCR of each life stage shows the relative amount of amplified receptor transcripts (Panel 1) in comparison to amplified tick β-actin transcripts (Panel 2). PCR products were obtained using single-stranded cDNA as the template. Note the higher intensity of receptor products in larvae and females. (B) Agarose electrophoresis of amplified receptor PCR products preparatory for Southern blot (Panel 1). Panel 2: Autoradiograph of the Southern blot of DNA in panel 1. Lane 1: negative control reaction (–) containing control cDNA not related to the receptor (Superscript preamplification system, Life Technologies). Lanes 2–5: PCR products corresponding to amplified receptor fragments from each life stage. N, nymphs; L, larvae.

Figure 3. Kyte and Doolittle hydrophilicity plots of (A) the *B. microplus* leucokinin-like peptide receptor (B) the *L. stagnalis* lymnokinin receptor, and (C) the *D. melanogaster* CG10626 gene product. Negative values indicate hydrophobic regions of the protein. All receptors show a similar structure of seven putative transmembrane regions, characteristic of GPCRs. The figure was created with DNASTAR software.

The *Drosophila* sequence shows higher homology to the *B. microplus* receptor than to the lymnokinin receptor, as expected due to the closer phylogenetic distance between Acari (ticks and mites) and insects than molluscs (Fig. 2). Thus, CG10626 putatively represents the first known insect leucokinin receptor. The *Drosophila* Malpighian tubule provides a model system for the analysis of neurohormonal control in insects (Dow *et al.*, 1998). The identification of

CG10626 as a putative leucokinin-like peptide receptor gene and the availability of a recently isolated leucokinin from *D. melanogaster* (Terhzaz *et al.*, 1999) will allow testing of the functional correspondence of this fruit-fly peptide and receptor. If confirmed, this receptor–peptide pair will constitute a system in which to analyse structure–activity relationships for leucokinins.

The similarity between the *B. microplus* receptor and the lymnokinin receptor is greater than that found among the subtypes of the mammalian NPY receptor, Y1, Y2 and Y4, which are activated by the same ligand (Larhammar, 1996). Thus, the ligand for the *B. microplus* receptor is most likely closely related to lymnokinin, PSFHSWS-NH₂, because GPCRs which interact with closely related ligands have the greatest sequence homology and structural conservation (Iismaa *et al.*, 1995; Cox *et al.*, 1997). However, leucokinins have not been identified in the Acari, but they have been isolated from insects (Nässel, 1996) and the pond snail (Cox *et al.*, 1997). There is evidence for their

presence in many invertebrates (Smart *et al.*, 1993; Elekes *et al.*, 1994; Schmid & Becherer, 1996). Thus, it is not unexpected that leucokinin-like peptides would also occur in ticks. Nevertheless, cloning of this *B. microplus* receptor constitutes the first indication for the presence of leucokinin-type hormone signalling in the Acari.

The lymnokinin receptor was identified as a leucokinin-like peptide receptor primarily by its mediation of leucokinin-induced intracellular calcium release (Cox *et al.*, 1997). This receptor was the first member of the leucokinin receptor subfamily, and thus it was not possible to identify conserved structural motifs that define this subfamily. However, with three members of this subfamily now known, it is possible to make some comparisons. The C-terminal end of TM II (residues 103–110 in the *B. microplus* receptor) and the first extracellular loop (residues 111–123) are highly conserved between the three leucokinin-like peptide receptors but not in the other neuropeptide receptor subfamilies (Fig. 2). Similarly, the first eight residues of the C terminus (337–344) are highly conserved among the leucokinin-like peptide receptors, and less conserved among the other neuropeptide receptors. It is interesting to note that TM VII is also highly conserved among all the compared neuropeptide receptors (Fig. 2).

The *B. microplus* receptor has two glycosylation sites in the N terminus, Asn 30 and Asn 41, and one in the second extracellular loop, Asn 204 (Fig. 1). Multiple glycosylation sites are found in the N termini of several neuropeptide receptors (Li *et al.*, 1992; Monnier *et al.*, 1992; Watson & Arkinstall, 1994; Cox *et al.*, 1997; Tensen *et al.*, 1998a,b). Two cysteines, Cys 348 and Cys 350 in the C-terminal region of the *B. microplus* receptor are potential sites for palmitoylation, which may be important for receptor function (Watson & Arkinstall, 1994; Iismaa *et al.*, 1995). Receptor binding sites for peptides and protein agonists of GPCRs include the N terminus and extracellular loops (Gether & Kobilka, 1998). The *B. microplus* receptor has several lysine (5) and arginine (4) residues in the extracellular loops, similar to residues found in the Y1 receptors that are involved in ligand binding (Berthold & Bartfai, 1997).

Among arthropods, ticks rank second only to mosquitoes as vectors of human disease (Bowman *et al.*, 1996). The expression of tick receptor mRNA throughout all life stages indicates that the receptor protein may be required for critical functions. Our discovery presents a target for the development of novel specific acaricides. These may prove useful to the cattle industry and in the prevention of transmission of human diseases, such as Lyme disease. This is the most common vector-borne disease in the USA, which is transmitted by closely related ixodids (Dolan *et al.*, 1997). GPCRs have proven to be among the most successful drug targets, and orphan or novel receptors have great potential for drug discovery (Stadel *et al.*, 1997). There is a precedent

for the successful targeting of arthropod GPCRs. Formamidines, such as amitraz, are synthetic acaricides that act on the octopamine receptor, which is only present in arthropods (Baxter & Barker, 1999). As all feeding stages of the tick remain on the host, our discovery also presents a target for the development of novel immunological approaches against this tick. Vaccines against gut antigens of *B. microplus* produce a protective immune response in cattle that reduces the number and the fecundity of ticks feeding on immunized cattle (De Rose *et al.*, 1999). Immunoglobins cross the midgut epithelium and enter the haemolymph of blood-feeding arthropods without losing their immunological properties, so there is a possibility that many cell membrane receptors could serve as targets for these vaccines (Sauer *et al.*, 1994). The difficulty in exploiting this strategy has been the lack of identification of essential tick receptors (Sauer *et al.*, 1994). The usefulness of the leucokinin-like receptor for preimmunizing cattle against *B. microplus* can now be explored.

Experimental procedures

Ticks

Southern cattle ticks (*B. microplus*) were from the Gonzalez strain, a pesticide-susceptible strain that is maintained at the Cattle Fever Tick Research Laboratory, USDA-ARS, Mission, TX, USA. This strain was originally obtained from an outbreak in Zapata County, TX, in 1994. Eggs were collected after engorged females were allowed to oviposit in a humidified incubator at 24 °C. Unfed larvae were collected after being raised in the incubator for 10 days. Nymphs were obtained by placing larvae on cattle, then collecting them 10 days after their nymphal moult. Adults were also raised on cattle and collected 15 days after their final moult. Ticks were frozen and stored at −80 °C until use.

cDNA synthesis and cloning

Total RNA was purified from tick larvae using TRIzol Reagent (Life Technologies, Gaithersburg, MD) following the manufacturer's protocol. Poly A^+ RNA was purified from the total RNA using an Oligotex mRNA Kit (Qiagen, Santa Clarita, CA). Double-stranded cDNA was synthesized using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA). PCR reactions were conducted in a PTC-200 Peltier Thermal Cycler or PTC-100 Programmable Thermal Controller (MJ Research Inc., Watertown, MA). Oligonucleotide primers were synthesized by Genosys Inc. (Houston, TX). All reactions were carried out in 50 μl volumes containing Clontech KlenTaq polymerase (1 μl), Clontech 10× cDNA PCR Reaction Buffer (5 μl), 0.2 mM each of four dNTPs and primers to final concentrations of 1 μM (degenerate) or 0.2 μM (specific primers) each. Marathon cDNA was used as the template for PCR with two degenerate oligonucleotide primers. Negative control reactions contained only one primer of the pair. These primers correspond to DNA sequences within the conserved regions of transmembrane regions III and VI of many GPCRs (Cox *et al.*, 1997): sense 5'-CCG GAT CCG (CT)(GC)A T(CT)(GA) (GC) (GC)I T(GT)G AC(CA) G(GC)T A-3' and antisense 5'-ACG AAT TCG G(GC) (CA) ICC A(GA)C AGA I(GC)(GA) (CT)(GA)A A-3'. PCR conditions were:

initial denaturation at 94 °C for 1 min followed by eight cycles of 94 °C for 20 s, and an annealing/extension step of 72 °C for 1.5 min that was decreased by 0.5 °C per cycle, followed by forty cycles of 94 °C for 20 s (denaturation), 68 °C for 1.5 min (annealing/extension) then a final extension of 5 min at 68 °C.

Rapid amplification of cDNA ends polymerase chain reaction

Four sequence specific primers were designed to amplify the 5' and 3' ends of the cDNA in RACE PCR. Sense primer sequences were: 1F (5'-AGT TCA TCA TCT GCG GTA TCT GGA C-3'); 2F (5'-GTC TCA CGC GCT CAA CCT GAC TAA G-3'). Antisense primers: 3R (5'-CAG ACA CAC GAG GAC GTG GTT ATA G-3'); 4R (5'-GTA CAC GAA ACA GAT GGT GAG CAG C-3'). Primers 2F and 3R corresponded to sequence regions interior to those of primers 1F and 4R, respectively. During synthesis of Marathon cDNA, adaptors were ligated to the ends of all cDNAs. Primers corresponding to the adapter sequence AP1 (external) and AP2 (internal) are supplied with the Marathon kit. Primers 1F and AP1 were used in the initial 3' RACE PCR. Primers 4R and AP1 were used in 5' RACE.

Initial RACE products were diluted and amplified in a nested 3' RACE reaction with 2F and AP2 primers and a nested 5' RACE reaction with primers 3R and AP2. Final primer concentrations were 0.2 μM in 50 μl volumes. Cycling parameters for the initial RACE were: 94 °C for 1 min, five cycles of 94 °C for 20 s and 72 °C for 1.5 min, five cycles of 94 °C for 20 s and 70 °C for 1.5 min, twenty-six cycles of 94 °C for 20 s and 68 °C for 1.5 min, and a final extension step of 68 °C for 5 min. Cycling parameters for the nested RACE reactions were: 94 °C for 1 min, twenty-five cycles of 94 °C for 20 s and 70 °C for 1.5 min, and a final extension step of 68 °C for 5 min. Products were separated on an agarose gel and bands of the sizes 2.0 kb from the 3' reaction and 0.8 kb from the 5' reaction were cut. The DNA was extracted using the Qiagen Quickgel extraction kit. PCR products were cloned into pCR 2.1 or pCR-TOPO plasmid vectors (Invitrogen, Carlsbad, CA) and electroporated into Electrocomp Topo 10F' cells or heat-shocked into Ultracomp Topo 10F' cells (Invitrogen), respectively. Positive colonies were selected with blue–white screening and plasmids were purified using Wizard Plus Minipreps (Promega Corp., Madison, WI).

Sequencing reactions were performed with AmpliTaq DNA polymerase and fluorescent dideoxynucleotides according to manufacturer's protocols (Applied Biosystems, 1998), and the reaction products were electrophoresed and analysed on an automated DNA sequencer (Applied Biosystems model 373) by the Gene Technologies Laboratory at Texas A & M University. Sequences were analysed using DNASTAR software (DNASTAR, Inc., Madison, WI).

Semiquantitative reverse transcription polymerase chain reaction

Methods used for semiquantitative RT-PCR were similar to those described by Dozois *et al.* (1997). Whole tick samples (50 mg) of each life stage (eggs, larvae, nymphs and adults of both sexes) were ground under liquid nitrogen with a mortar and pestle. Poly A$^+$ RNA was purified from the ground samples using a Dynabeads' mRNA Direct kit (Dynal, Oslo, Norway). First strand cDNA was synthesized from one tenth of the Poly A$^+$ RNA at 42 °C for 50 min with oligo(dT)$_{12-18}$ primer using Superscript II Reverse transcriptase (Superscript Preamplification System, Life Technologies). PCR amplification of β-actin cDNA was performed using primers Act-3F, 5'-TCC TCG TCC CTG GAG AAG TCG TAC-3', and Act-4R,

5'-CCA CCG ATC CAG ACC GAG TAC TTC-3' specific to the *B. microplus* β-actin gene. The gene sequence was obtained by PCR using primers that correspond to conserved regions of insect actin genes (H. He, unpublished). Reactions contained one-tenth (2 μl) of the synthesized cDNA, 200 μM each of four dNTPs, 0.2 μM of each primer, 1 μl Taq polymerase, and 1× reaction buffer (Boehringer Mannheim) in a final volume of 50 μl. Taq was added after the reaction was brought to 94 °C. The following cycling parameters were used: 94 °C for 1 min followed by twenty-five cycles of 94 °C for 20 s, 62 °C for 30 s, and 72 °C for 45 s. PCR amplification of receptor cDNA was performed using primers designated SC2-F, 5'-CTC CGG GAA GTT TTC CTA AAG A-3', and SC3-R, 5'-TGG TGG TTG GAC TCA AAT TAC AC-3'. PCR conditions were identical to the actin PCR amplification except that thirty-five cycles were used to amplify the receptor cDNA. Five microlitres of each actin reaction and 20 μl of each receptor reaction were electrophoresed on 1% TBE agarose gels containing ethidium bromide. Gels were photographed with Polaroid film and the images were scanned with a Hewlett Packard ScanJet 3c. The intensity of the bands was determined using Kodak Digital Science 1D software (Kodak Scientific Imaging Systems, New Haven, CT). The relative level of receptor mRNA in each tick stage was indirectly estimated by the ratio of the intensity of the receptor band to that of the actin band.

Southern blot

To assure that the RT-PCR products corresponded to the cloned receptor, each was diluted 1 : 500 and reamplified in a 100 μl reaction using the same reagents and concentrations as the original RT-PCRs. The following cycling parameters were used: 94 °C for 1 min followed by forty cycles of 94 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s. Twenty microliters of each reaction was run on a TBE 1% agarose gel that was photographed. DNA was transferred to S & S Nytran membrane (Schleicher and Schuell, Keene, NH) using standard upward blotting techniques following alkaline denaturation (Ausubel *et al.*, 1995). After transfer, the blot was baked at 80 °C for 30 min, then UV crosslinked for 3 min on a transilluminator.

In order to unequivocally confirm that the amplified products were receptor specific, a DNA receptor fragment (base pairs 986–1304) (Fig. 1) was cloned and sequenced to serve as a template for a radiolabelled probe for Southern blots. For this, the Ambion DECAprime II Random Priming DNA Labelling kit (Ambion, Austin, TX) was used with α-^{32}P dCTP (NEN Life Science Products, Boston, MA). The blot was allowed to hybridize overnight at 42 °C in ULTRAhyb' (Ambion), then washed 2 × 5 min in 1× SSC, 0.1% sodium dodecyl sulphate (SDS) at 42 °C then 2 × 15 min in 0.1× SSC, 0.1% SDS at 50 °C. The blot was then exposed to Kodak Biomax ML film (Eastman Kodak, Rochester, NY).

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Systematic G-protein-coupled Receptor Analysis in *Drosophila melanogaster* Identifies a Leucokinin Receptor with Novel Roles*

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Leucokinins are insect neuropeptides that stimulate hindgut motility and renal fluid secretion. *Drosophila* has a single leucokinin gene, *pp*, encoding the longest known leucokinin, Drosokinin. To identify its receptor, a genome-wide scan for G-protein-coupled receptors was performed *in silico* and candidate receptors identified by similarity to known tachykinin receptors. The deduced peptides were expressed, with a transgene for the calcium reporter aequorin, in S2 cells and only one gene (*CG10626*) encoded a protein that responded to Drosokinin. The properties of the heterologously expressed receptor (action through intracellular calcium with an EC_{50} of 4×10^{-11} M and a $t_{1/2}$ <1 s) match closely those reported for the action of Drosokinin on Malpighian (renal) tubules. Antibodies raised against the receptor identified known sites of leucokinin action: stellate cells of the Malpighian tubule, two triplets of cells in the *pars intercerebralis* of the adult central nervous system, and additional cells in larval central nervous system. Western blots and reverse transcription-PCR confirmed these locations, but also identified expression in male and female gonads. These tissues also displayed elevated calcium in response to Drosokinin, demonstrating novel roles for leucokinin. A functional genomic approach has thus yielded the first complete characterization of a leucokinin receptor in an insect.

The completion of genome projects, such as that of *Drosophila melanogaster* (1), has given a unique opportunity to screen *in silico* for entire gene families. We have used this approach to identify a neuropeptide receptor implicated in renal function via a systematic scan of the G-protein-coupled receptor (GPCR)[1] superfamily. The production of primary urine in the Malpighian tubules of insects is driven by the action of diuretic factors. The ability of these hormones and their receptors to regulate fluid and ion secretion make them attractive targets for novel insect control approaches. The insect myokinins, known as leucokinins, are one such group of neuropeptides. They were first isolated from the cockroach *Leucophaea maderae* as a family of small peptides that stimulate hindgut motility (2–6). They are now known to be present in many

other insect species as well as parasitic nematodes, crustaceans, and molluscs (7–15) (Table I). The leucokinins vary from 6 to 15 amino acids in length and are characterized by a C-terminal pentapeptide motif (F*XX*WG-amide) essential for biological activity.

In *Drosophila* it has been shown that leucokinins act to stimulate fluid secretion by raising the chloride shunt conductance (16), not via cyclic nucleotide signaling but via an increase in intracellular calcium levels (7, 17). Furthermore this increase occurs only in the smaller secondary stellate cells (7). No leucokinin receptors have been identified in insects, although a receptor for a distantly related peptide, that shares a FxSWx-amide motif with Drosokinin, has been cloned from the pond snail *Lymnaea stagnalis*, and was found to be a member of the GPCR superfamily (15). A further leucokinin-like receptor has been cloned in the cattle tick *Boophilus microplus*, and this also displays the seven-transmembrane domain structure characteristic of GPCRs (18). Although a *Drosophila* gene has been identified as being similar to the lymnokinin receptor *in silico* (18, 19), no functional evidence for its identification as a leucokinin receptor is available nor is the similarity between *Drosophila* and *Lymnaea* leucokinins compelling (Table I). Accordingly, in this paper, we used a genome-wide scan to identify the clade of GPCRs that were most similar to receptors for tachykinins, including the lymnokinin receptor, and to assay them all systematically for activity. From this group a single gene, *CG10626*, was shown to encode a functional leucokinin receptor, with functional properties consistent with those observed in intact renal tubules and expression patterns that map closely to those predicted for such a receptor. This is the first functionally characterized example of an insect leucokinin receptor gene.

MATERIALS AND METHODS

Fly Rearing

Oregon R (wild-type) flies were reared in vials or bottles on standard *Drosophila* cornmeal yeast agar medium at 23 °C in a 12 h light:12 h dark cycle. Flies transgenic for the aequorin transgene, under control of the UAS promoter (UAS-aeq) enhancer trap line that directs GAL4 expression to secondary stellate cells (c710) and a heat-shock/GAL4 construct (hsGAL4), were those described previously (7, 20, 21).

Data Mining

The Berkley *Drosophila* Genome Project data base (www.fruitfly.org) was searched with representative GPCR protein sequences and putative *Drosophila* GPCRs translated to protein sequences and used to verify the automated translations provided by the genome project, where available. Dendrograms were created in Clustal X (22) using amino acid sequences and visualized using TreeView (23). Sequence alignments, hydrophobicity, transmembrane, and antigenicity plots were created in MacVector (Accelrys). The web-based programs Tmpred (24), TMHMM (25), SOSUI (26), PredictProtein (27), and HMMTOP (28) were also used for transmembrane prediction. The cis-analyst search tool at the Berkley *Drosophila* Genome Project web site was used to identify clustered transcription factor binding sites (29).

TABLE I
Members of the insect leucokinin family (adapted from Ref. 7)
Residues identical to those of Drosokinin are in bold.

Species	Number	Sequence	Effect on *D. melanogaster* tubules	Ref.
D. melanogaster	1	NSVVLGKKQRFHSWGamide	Yes	7
Aedes aegypti	1	NSKYVSKQKFYSWGamide	Yes	12
	2	NPFHAWGamide	No	12
	3	NNPNFYPWGamide	Yes	12
Culex salinarius	1	NPFHSWGamide		13
L. maderae	1	DPAFNSWGamide	Yes	3
	2	DPGFSSWGamide	No	3
	3	DQAFNSWGamide	No	4
	4	DASFHSWGamide	Yes	4
	5	GSGFSSWGamide	No	5
	6	pESSFHSWGamide	Yes	5
	7	DPAFSSWGamide	Yes	6
	8	GASFYSWGamide	No	6
Locusta migratoria	1	AFSSWGamide		11
Acheta domesticus	1	SGADFYPWGamide		10
	2	AYFSPWGamide		10
	3	ALPFSSWGamide		10
	4	NFKFNPWGamide		10
	5	AFHSWGamide		10
Pennaeus vannamei	1	ASFSPYGamide		35
	2	DFSAWAamide		35
Lymnaea stagnalis	1	PSFSSWSamide		15

Genomic DNA Preparation

30 Oregon R flies were frozen in liquid N_2, then homogenized in 200 μl of buffer (100 mM Tris-HCl, pH 7.5, 100 mM EDTA, 100 mM NaCl, 0.5% w/v SDS). A further 200 μl of buffer was added, then incubated at 65 °C for 30 min. 800 μl of 1.4 M Kac, 4.3 M LiCl was added and incubated on ice for 10 min, followed by centrifugation at 13,000 rpm. 1 ml of supernatant was transferred to a clean tube avoiding debris, and 600 μl of isopropyl alcohol added. This was mixed and spun for 15 min at 13,000 rpm. Supernatant was aspirated off and washed with 70% ethanol, spun again for 5 min, and the DNA pellet dried. The pellet was resuspended in 150 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and 1 μl was used as template in PCR reactions.

RT-PCR

Tissue-specific poly(A)$^+$ RNA was extracted (Dynal mRNA DIRECT kit) and reverse-transcribed with Superscript Plus (Invitrogen) as described previously (30). In the case of developmental stages, 5 μg of poly(A)$^+$ RNA was reverse-transcribed directly with Superscript Plus using an oligo(dT) primer. 1 μl of the reverse transcription reaction was used as template for each PCR reaction; intron-spanning gene-specific primers were used for the amplification of cDNA or genomic DNA template. Forward primers (CATCGTCTGGTTTTGCTGCG) and backward primers (CCATTGACATTGTTGTTCTGCCC) were expected to generate a product of 360 bp with cDNA templates and 1 693 bp with genomic DNA template. Primers were used at 2.5 μM in a final volume of 25 μl, using the following cycle conditions: 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 56 °C for 15 s, 72 °C for 30 s, and 72 °C for 5 min.

Expression Constructs

Primers were designed for each candidate receptor to allow amplification from the start to the stop codons of each ORF. Forward primers were designed to include a 5′ Kozak translational initiation sequence (G/ANNATGG; Ref. 44). Amplification was carried out with EXPAND High Fidelity Polymerase (Roche Molecular Biochemicals) according to manufacturer's instructions. For amplification of the *CG10626* ORF, forward (GACATGGACTTAATCGAGCAGGAG) and reverse (TTA-AAGTGGTTGCCACAAGGAC) primers were used to generate a fragment of 1626 bp. Oregon R cDNA was used as template. For amplification of apo-aequorin ORF, forward (GCAAACATGACAAGCAAACA-ATAC) and reverse (TTAGGGGACAGCCTCCACCGTAGAG) primers were used to generate a fragment of 597 bp. Genomic DNA from ae-quorin expressing flies (31) was used as template. PCR products were purified by gel extraction and directly cloned into the pMT-V5/His TOPO TA-inducible expression vector (Invitrogen). Constructs were verified by restriction enzyme digestion and automated sequencing.

S2 Cell Culture

S2 cells were maintained in DES medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen). Cells were grown in suspension at 23 °C at an initial density of 2–4 × 10^6 cells/ml. S2 cells were transiently transfected at a density of 1 × 10^6 cells/ml using a calcium phosphate transfection kit (Invitrogen); procedures were carried out according to the manufacturer's instructions. Cells were co-transfected with 10 μg of pMT-aeq (an expression vector containing an inducible apo-aequorin gene) and 10 μg of the relevant receptor expression construct. Cells were used 24 h post-induction.

Real-time Luminescent Measurements of Intracellular Calcium Using Aequorin

Aequorin is widely used as a transgenic reporter for calcium (32) and in *Drosophila* can be directed to specific cells using the GAL4/UAS binary reporter system (20). It has previously been used in the *Drosophila* tubule to show that the neuropeptide CAP$_{2b}$ acts to raise Ca^{2+} in only principal cells (20) and leucokinin only in stellate cells (7, 16, 20).

S2 Cells

Transiently transfected S2 cells were harvested and incubated with 2.5 μM coelenterazine (Molecular Probes) in the dark at room temperature for 1 h. 25,000 cells were used per sample tube in DES medium supplemented with 10% fetal calf serum. The neuropeptides Drosokinin (NSVVLGKKQRFHSWGamide) (7) and CAP$_{2b}$ (pyro-ELYAFPRV-amide) (17) were synthesized by Research Genetics. Peptide agonists were diluted to working concentration in DES medium/fetal calf serum. Peptide was injected into each sample and the responses measured over a period of 4 min. At the end of each experiment the total luminescence of each sample was assessed by injecting 100 mM Ca^{2+}, 1% Triton X-100 to permeabilize cells and so to discharge the remaining aequorin. Measurements were carried out with a Berthold-Wallac luminometer. Calcium concentrations were calculated as described previously (20) by reverse integration with a program written in Perl and data analyzed using Excel (Microsoft) and Cricket Graph.

Intact Tissue

Experiments were carried out as described previously (7, 16, 20). Tissues were dissected from adult (3–10 days old) flies or, in the case of larval central nervous system, from 3rd instar larvae. Tubules were dissected from c710 UAS-aeq flies. For other tissues, specific GAL4 driver lines were not available, and so aequorin was ubiquitously expressed under heat-shock control. Hindgut and larval central nervous system were dissected from hsGAL4 UAS-aeq flies/larvae that had been previously heat-shocked twice at 36 °C for 30 min at 24-h intervals. Either 80 tubules, 20 hindguts, or 10 larval central nervous systems were used per sample and incubated in Schneider's medium (Invitrogen) containing 2.5 μM co-elenterazine for 3 h. Drosokinin was diluted in Schneider's medium such that the final applied concentration was 10^{-7} M. At the end of the experiment the total luminescence of each sample was assessed by injecting 100 mM Ca^{2+}, 1% Triton X-100.
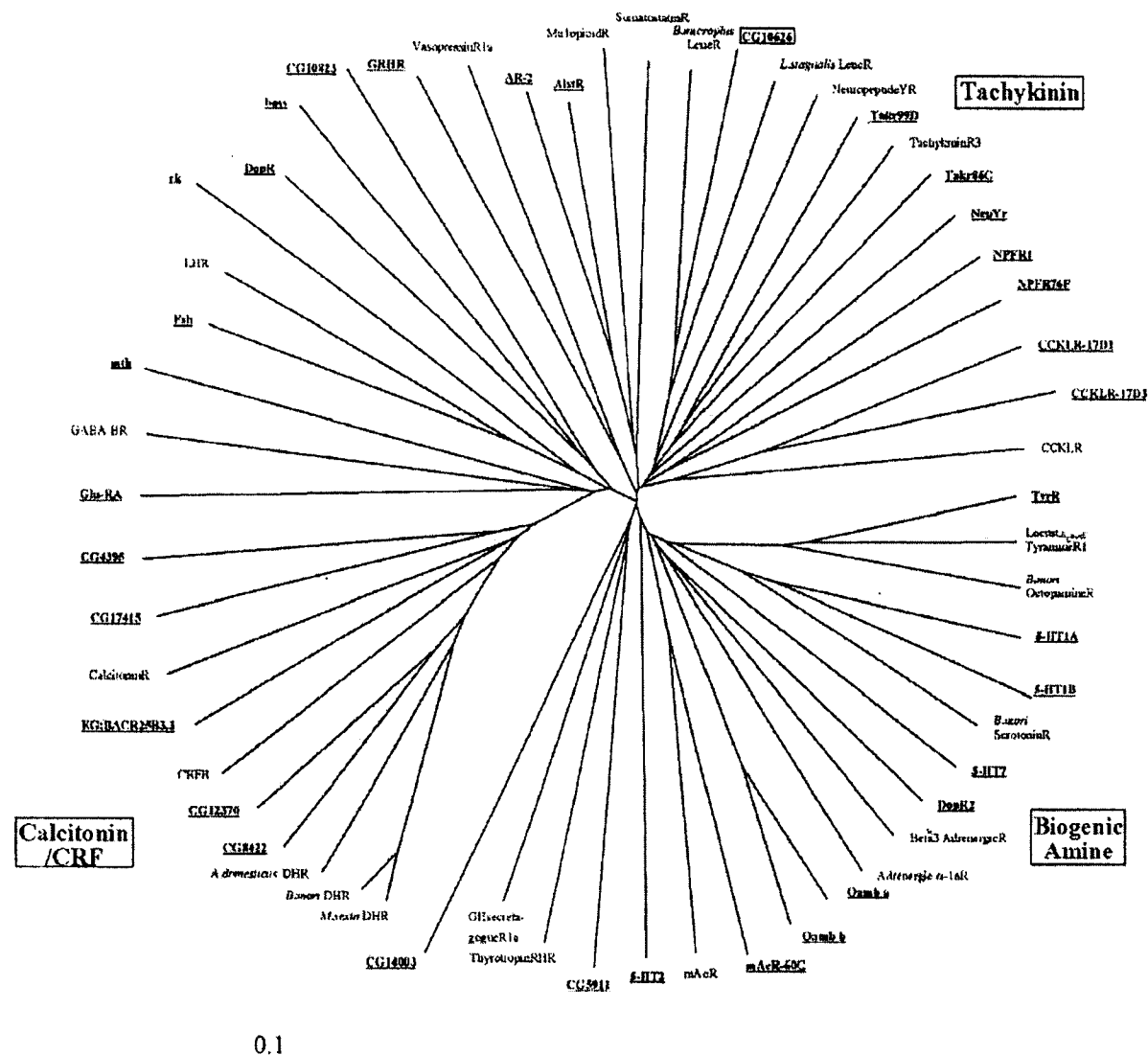
0.1

FIG. 1. **Dendrogram of GPCRs.** *Drosophila* neuropeptide receptors are mapped together with representatives of other *Drosophila* GPCR groups, other insect GPCRs, and vertebrate (human) GPCRs. *Drosophila* receptors are underlined, and CG10626 is boxed. Unless otherwise stated, non-*Drosophila* receptors are human. The tachykinin-like, biogenic amine-like, and diuretic hormone-like receptor families are highlighted.

Measurements were carried out with a Berthold-Wallac luminometer and calcium concentrations calculated as above.

Peptide Antibody Production

The putative CG10626 amino acid sequence was scanned for highly antigenic regions and a 14-amino acid segment identified (GIYNGSS-GQNNNVN) contained within the C terminus of the protein sequence. This showed no significant similarity to any other *Drosophila* sequences in the genome. An anti-peptide antibody was generated in rabbit by Genosphere Biotechnologies (Paris, France).

Whole Mount Immunocytochemistry

(i) *Intact Malpighian Tubules*—Whole tubules were fixed, permeabilized, and stained as described previously (36). Rabbit polyclonal anti-CG10626 peptide antibody (see above) was used diluted 1:1000. Secondary antibody used was donkey fluorescein-labeled anti-rabbit IgG (Diagnostics Scotland), used at 1:250 dilution.

(ii) *S2 Cells*—Coverslips were coated with poly-L-lysine solution (100 μg/ml) for 30 min, washed with water, and allowed to dry. S2 cells were added at a density of 6 × 10⁶ cells/ml and left for 15 min to allow cells

to settle and adhere. Excess solution was removed and the coverslips washed twice in PBS. Cells were fixed by the addition of methanol (−20 °C) and incubated for 4 min at −20 °C. Cells were washed three times in PBS and blocked in PBS/0.2% (w/v) BSA for 5 min. Cells were incubated for 25 min with rabbit polyclonal anti-CG10626 peptide antibody diluted 1:1000 in PBS/BSA. They were then washed three times in PBS/BSA and incubated for 25 min with donkey fluorescein-labeled anti-rabbit IgG antibody diluted 1:400 in PBS/BSA. This was followed by three PBS/BSA washes and three final 5-min washes in PBS alone. Coverslips were then mounted on slides using Vectashield mounting medium (Vector) and a coverslip sealed on with glycerol-gelatin (Sigma). Slides were viewed by epifluorescence, using a fluorescein filter set.

(iii) *Larval and Adult Central Nervous System*—Adult or larval central nervous system was dissected and fixed in 8% paraformaldehyde for 20 min (adult) or 2 h (larval) at room temperature. They were washed six times in washing buffer (0.15 M sodium phosphate, 0.1% w/v sodium azide, 0.5% v/v Triton X-100, pH 7.4), followed by blocking in blocking buffer (washing buffer, 10% v/v goat serum) for 2 h. Samples were incubated overnight at 4 °C with rabbit polyclonal anti-CG10626

peptide antibody diluted 1:1000 in blocking solution, washed for 2 h in washing buffer, and blocked again for 4 h. Samples were incubated overnight at 4 °C with either donkey fluorescein-labeled anti-rabbit IgG (Diagnostics Scotland) or goat Texas Red-labeled anti-rabbit IgG (Abcam), diluted 1:250 in blocking buffer. Samples were washed at room temperature for 1 h in washing buffer, followed by incubation for 15 min each in 20, 40, 60, and 80% glycerol in 0.04 M sodium carbonate buffer, pH 9.4. Finally, tissues were mounted on a slide with Vectashield (Vector) and a coverslip sealed on with glycerol-gelatin (Sigma). Slides were viewed by epifluorescence, using fluorescein or rhodamine filter sets as appropriate.

Western Blot Analysis

Protein samples were prepared from whole tissue by homogenization in Tris lysis buffer (70 mM Tris, 2% (w/v) SDS) with protease inhibitor mixture (Sigma, P-8340). Samples were centrifuged for 10 min at 13,000 rpm at 4 °C to remove debris. Supernatant was removed to a clean tube. S2 cells were pelleted by centrifugation at 1000 rpm for 2 min, washed in PBS, pelleted again, and resuspended in cell lysis buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 1% (v/v) Igepal CA-630 (Sigma)) with protease inhibitor mixture (Sigma, P-8340). Cell lysates were incubated at 37 °C for 10 min, vortexed, and centrifuged at 13,000 rpm for 10 min to remove cell debris. Supernatant was transferred to a new tube. Samples were run on SDS-PAGE and blotted according to standard methods. The filter was blocked for 3 h in PBS with 0.1% Tween 20 and 10% nonfat dry milk and washed in PBS/Tween 20 once for 15 min then three times for 5 min. The filter was incubated overnight at 4 °C with primary antibody diluted 1:1 000 in PBS/Tween 20/milk, then washed in PBS/Tween 20 once for 15 min and three times for 5 min, then incubated for 2 h with secondary antibody (1:2500 horseradish peroxidase-labeled anti-rabbit IgG antibody (Amersham Biosciences)) diluted in PBS/Tween 20/milk. Final wash was in PBS for 3 h; protein bands were visualized using enhanced chemiluminescence (ECL, Amersham Biosciences).

Deglycosylation

20 μg of either transfected S2 cell or tubule protein extract was denatured in 1 × denaturing buffer (0.5% SDS, 1% β-mercaptoethanol) at 100 °C for 10 min. 0.10 volume of 0.5 M sodium phosphate, pH 7.5 at 25 °C, 10% Nonidet P-40, and 2500 units of peptide:N-glycosidase F (New England Biolabs), an enzyme that removes N-glycosylation, were added and incubated for 16 h at 37 °C. 15 μg of each sample was then analyzed by Western blotting.

Expression in Escherichia coli

The LKR ORF was cloned into the pCR T7/NT TOPO TA vector (Invitrogen). The ORF was amplified using the same primers as for S2 cell expression. The construct was transformed into competent *E. coli* BL21/pLys cells. Single colonies were grown overnight in 5 ml of L broth, 100 μg ml⁻¹ ampicillin, 34 μg ml⁻¹ chloramphenicol at 37 °C. 5 ml of culture medium were seeded with 250 μl of overnight culture and grown to OD_{600} 0.5–0.8 at 37 °C. The culture was then induced with 1 mM isopropyl-β-D-thiogalactopyranoside and grown at room temperature for 4 h. 1 ml of cells was pelleted (13,000 rpm, 1 min) and resuspended in 150 μl of lysis buffer (50 mM potassium phosphate, pH 7.8, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 10 mM imidazole). Cells were lysed by three cycles of freeze-thawing (freeze in liquid N_2, thaw at 42 °C) and whole cell lysate analyzed on a SDS-PAGE gel.

RESULTS

Identification of a Drosokinin Receptor—All GPCRs were identified in the *D. melanogaster* genome sequence by BLAST similarity searches with known GPCRs; putative translations were deduced and assembled into a dendrogram by sequence alignment (Clustal X (22) and Treeview (23)) (Fig. 1). Similar results have been reported elsewhere (19, 37, 38). The two non-insect leucokinin-like receptors (from *L. stagnalis* (15) and *B. microplus* (18)) were included in the dendrogram and were found to sit within a tightly defined clade, containing characterized examples of *Drosophila* tachykinin receptors (40–42), together with novel putative genes. As leucokinins are considered to be distantly similar to tachykinins, this was clearly the group containing the strongest candidates for a leucokinin receptor.
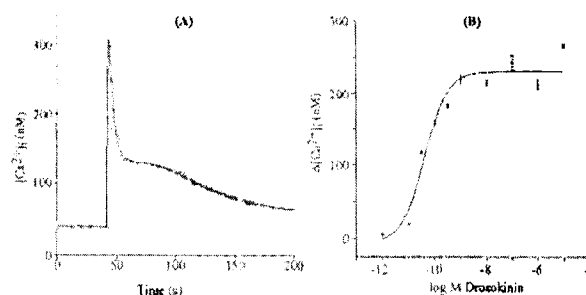

FIG. 2. **CG10626 encodes a functional leucokinin receptor that acts through intracellular calcium.** *A*, real-time measurements of intracellular calcium in S2 cells. $[Ca^{2+}]_i$ increases in *DLKR* ORF and apo-aequorin ORF transfected S2 cells induced by 10^{-7} M Drosokinin. Data are expressed as $[Ca^{2+}]_i$ (nM) against time (s); each data point corresponds to 0.1 s. The *trace* shown is the average response from 10 independent readings; S.E. *bars* are shown for each data point. *B*, dose-response curve for Drosokinin in S2 cells transfected with *CG10626*. Drosokinin-stimulated $[Ca^{2+}]_i$ increases were measured in experiments performed as in Fig. 2*A*. Values were expressed as maximal (nM) − average background (nM) (mean ± S.E., *n* = 5–10). Where error bars are not visible, they are too small to reproduce.

S2 cells were transiently transfected with both putative receptor ORFs and the apo-aequorin ORF. Only cells transfected with the CG10626 ORF responded in a dose-dependent manner to Drosokinin (Fig. 2*A*): no response was seen in cells transfected with the closely related *Drosophila* neuropeptide receptors that sat within the same subgroup of the dendrogram (NepYr, NPFR1, NPFR76F, Takr86C, and Takr99D; data not shown). In cells transfected with *CG10626*, intracellular calcium levels were seen to increase from basal levels of 60 to 400 nM, a 5-fold increase. The calcium response is biphasic in nature, with an initial large calcium rise followed by a rapid exponential decay with evidence of a secondary response between 20 and 60 s post-stimulation. The initial response gave a sigmoidal dose-response curve with an EC_{50} value of 4.3 ± 0.16 × 10⁻¹¹ M (Fig. 2*B*). The nature of this response and the EC_{50} value are comparable with that of the effect of Drosokinin on intact Malpighian tubules (EC_{50} approximately 1 × 10⁻¹⁰ M) (7). As controls, *CG10626*/apo-aequorin co-transfected cells also did not respond to the *Drosophila* diuretic neuropeptide CAP_{2b}, nor did cells transfected with apo-aequorin alone respond to Drosokinin. Western blot analysis using an anti-aequorin antibody (Covalab) confirmed that the aequorin protein was strongly expressed in S2 cells when expression of the transgene was induced (data not shown). It is thus clear that *CG10626* encodes a functional leucokinin receptor. Accordingly, we renamed *CG10626* as Drosokinin receptor (*DLKR*).

Characterization of the DLKR Gene—The *DLKR* gene is localized at 64E1 on chromosome 3L, the open reading frame encoding a 540-amino acid protein, which has an estimated molecular mass of 60.6 kDa.

The *CG10626* ORF nucleotide sequence was used in a BLAST search against the *Drosophila* expressed sequence tag data base to identify a cDNA clone, RE03009, from the Riken embryo library (43). This clone was obtained from Research Genetics and sequenced in full on both strands (BaseClear). Sequencing of the insert suggests the clone contains the full mature transcript of this gene (Fig. 3*A*). This is a 2930-bp transcript containing the coding sequence (1626 bp), a 539-bp 5'-untranslated region and a 768-bp 3'-untranslated region. The sequence contains a perfect consensus sequence for the initiation of transcription (TCAGTT) beginning with the A at +1. There are two in-frame ATG start codons 6 bp apart, although the first does not have as good a consensus sequence for translational initiation, and so it is presumed the later ATG

Drosokinin Receptor

(A)

```
AGTTGACTTCGGGAGCTTTAATCGTGCGGTTGTATCTTGCGATCGAAACTGTATCGAAAAGATATTAAATATTCAGCAAAAAATTCGTTGGCAATAAGAT  100
ACTGAATTCGGCCCGTAGAATATTATAAAGATTTATTTGTGAAACAAATAACACTAATTAAAAACCTTTCAAAAGTGATTTTATCTAAGTGTATAATTAA  200
ACATATACACAGTTTACTGTTTGTTCGCCAACTGATTATGGCCATTTAGCAGATAAATGCAGAGTCAATAGCAAACTAGCAAACCAGAAAGCTAATTGCA  300
                                                                                              ><
TTGGGCAGTGAAAGAAATAAATGAGCATTTACTAATTAATTACACAAAAAAACGCATTTAAATCCATCAGAAGAGTAATTAATTTTTGTGGGTTAGATTG  400
CAATGAAGCTCAGAAGCCCCTGAAGATGTATTGCACTACATAGAGCAGCAGCATTAAATACAAGGTTTGAAGGATATCATATCCTCATTAGGA  500
TACACAACTAAAACTAAAAAAACGAAAAAGTGTTATGGCAATGGACTTAATCGAGCAGGAGTCCCGCCTGGAATTCCTGCCCGGAGCCGAGGAGGAAGCAG  600
                            M  D  L  I  E  Q  E  S  R  L  E  F  L  P  G  A  E  E  E  A   20
      ><
AATTTGAGCGTCTATACGCGGCTCCCGCTGAGATTGTGGCCCTGTTGTCCATTTCTATGGGGGAATCAGTATCGTGGCCGTCATTGGCAACACTTTGGT  700
E  F  E  R  L  Y  A  A  P  A  E  I  V  A  L  L  S  I  F  Y  G  G  I  S  I  V  A  V  I  G  N  T  L  V  54
CATCTGGGTGGTGGCCACGACCAGGCAAATGCGGACCGTGACAAATATGTATATCGCTAATTTGGCTTTTGCCGATGTGATTATTGGCCTCTTCTGCATA  800
 I  W  V  V  A  T  T  R  Q  M  R  T  V  T  N  M  Y  I  A  N  L  A  F  A  D  V  I  I  G  L  F  C  I  87
CCATTTCAGTTCCAGGCTGCCCTGCTGCAGAGTTGGAACCTGCCGTGGTTCATGTGCAGCTTCTGCCCCTTCGTGCAAGCCCTGAGTGTAAATGTCTCGG  900
 P  F  Q  F  Q  A  A  L  L  Q  S  W  N  L  P  W  F  M  C  S  F  C  P  F  V  Q  A  L  S  V  N  V  S  120
                                                           ><
TATTCACGCTGACCGCCATTGCAATCGATCGGCATAGGGCCATCATTAATCCACTTAGGGCACGTCCCACCAAGTTCGTATCGAAGTTCATAATTGGTGG  1000
 V  F  T  L  T  A  I  A  I  D  R  H  R  A  I  I  N  P  L  R  A  R  P  T  K  F  V  S  K  F  I  I  G  G  154
AATTTGGATGCTGGCCCTGCTATTTGCGGTGCCCTTTGCCATTGCCTTTCGTGTGGAGGAGTTGACCGAAAGATTTCGCGAGAACAATGAGACCTACAAT  1100
 I  W  M  L  A  L  L  F  A  V  P  F  A  I  A  F  R  V  E  E  L  T  R  R  F  R  E  N  N  E  T  Y  N  187
                                                           ><
GTGACGCGGCCATTCTGCATGAACAAGAACCTATCGGATGATCAATCATTTCGCTGGTTTTTGTGCAGTATCTGGTTCCATTCTGTG  1200
 V  T  R  P  F  C  M  N  K  N  L  S  D  D  Q  L  Q  S  F  R  Y  T  L  V  F  V  Q  Y  L  V  P  F  C  220
                                 ‡
TCATCAGCTTTGTCTACATCCAGATGGCCGGTACGATTGTGGGGCACACGTGCTCCTGGTAACGCACAGGATTCACGGGACATAACGCTGTTGAAAAACAA  1300
 V  I  S  F  V  Y  I  Q  M  A  V  R  L  W  G  T  R  A  P  G  N  A  Q  D  S  R  D  I  T  L  L  K  N  E  254
      ><
GAAGAAGGTCATCAAAATGCTGATTACGTGGTCATTATCTTTGGACTCTGCTGGCTGCCACTGCAGCTCTATAATATTCTGTATGTCACGATACCGGAA  1400
 K  K  V  I  K  M  L  I  I  V  V  I  I  F  G  L  C  W  L  P  L  Q  L  Y  N  I  L  Y  V  T  I  P  E  287
ATCAACGACTACCACTTCATTAGCATCGTCTGGTTTTGCTGCGATTGGCTGGCCATGAGCAATAGCTGCTACAATCCCTTTATTTATGGCATCTACAATG  1500
 I  N  D  Y  H  F  I  S  I  V  W  F  C  C  D  W  L  A  M  S  N  S  C  Y  N  P  F  I  Y  G  I  Y  N  320
AAAAATTTAAGCGGGAATTCAACAAGCGATTTGCGGCCTGTTTCTGCAAGTTCAAGACGAGCATGAACGCCCACGAAAGGACCTTTTCGATGCACACCCG  1600
 E  K  F  K  R  E  F  N  K  R  F  A  A  C  F  C  K  F  K  T  S  M  D  A  H  E  R  T  F  S  M  H  T  R  354
CGCCAGCTCCATAAGGTCAACCTACGCCAACTCCTCGATGCCGAATCCGGAGTAATCTCTTTGGTCCGGCGCGTGGTGGTGTCAACAATGGGAAGCCGGGC  1700
 A  S  S  I  R  S  T  Y  A  N  S  S  M  P  I  R  S  N  L  F  G  P  A  R  G  G  V  N  N  G  K  P  G  387
TTTGCATATGCCGCGCGTGCATGGATCCGGTGCTAACAGCGGCATTTACAACGGAAGTAGTGGGCAGAACAACAATGTCAATGGCCAACATCATCAGCATC  1800
 L  H  M  P  R  V  H  G  S  G  A  N  S  G  I  Y  N  G  S  S  G  Q  N  N  N  V  N  G  Q  H  H  Q  H  420
AAAGCGTGGTTACCCTTTGCGGCCACTCCGGGTGTTTGGGCACCAGGTGTTGGCGGTTGCAATGCCGCCGTGGCGGCGGGAAACAACTTCAAACCTCTGCATCC  1900
 Q  S  V  V  T  F  A  A  T  P  G  V  S  A  P  G  V  G  V  A  N  P  P  W  R  P  N  N  F  K  P  L  H  P  454
GAACGTAATCGAATGCGGAGGACGACGGTGGCACTCATGGAGCTGCCATCAACCACGCCCCCAGCGAGGAGTTGGCATCCGGGGCCGGAGTCCAGTTGGCC  2000
 N  V  I  S  C  E  D  D  G  H  A  H  E  L  P  S  T  P  P  S  E  E  L  A  S  G  A  G  V  Q  L  A  487
CTGCTAAGCAGGGAGAGCTCCAGCTGCATTTGCGAACAGGAATTTGGCAGCCAAACCGAATGCGATGGCACCTGCATACTCAGCGAGGTGTCGCGAGTCC  2100
 L  L  S  R  E  S  S  S  C  I  C  E  Q  E  F  G  S  Q  T  E  C  D  G  T  C  I  L  S  E  V  S  R  V  520
ACCTGCCCGGCTCGCAGGCGAAGGACAAGGATGCGGGCAAGTCCTTGTGGCAACCACTTTAATTTAAACTAGAGCGATCTAAGTTATAGCCGTGTTCCAA  2200
 H  L  P  G  S  Q  A  K  D  K  D  A  G  K  S  L  W  Q  P  L  *                                    540
TGACACATTCCGCAGCCAGTCGGCATTCACATATGATGTGTATTCCAGTAAATTGGCTGTCATAGCTCATTCATTTAACGTATGTGTGTTAATGTTTTAG  2300
TGAATGACTATTATTAGGGTATAGCTACTACACTAATATGTTTAAATTTTATTGTAATTGTATGTAAGGCTTTAGTAAAACCCCTTTACAGTGTTTAGAT  2400
ATCCCGCAACTAAATGTGTCATTGGAACATGACCATTTGCCTCTGTGATTTCGTCTTAATGAATTGTGATTACAAGTCCCATTGCTGATAATTGTTTCGC  2500
AGAAGGATATATATTGAATGAGTGTGAATGCTGGTCGTGAGCCGTGTACAGTATTATAATAATGGTATCTTCAAATTCGCATCCTTAAGCACGAAGTCC  2600
AAACGTTGTAAATAATCGATTCGTTAATGACCTTATCAAACTCAAACCAAAACTCACCAAATCAAATGTATTATTAGCTGAACAAAAAATGAATCAAATT  2700
AAATTTAGCACCTGAAATCAATTAAGTAGCATTAAATTAAATTATTTAAATAGTATTTTAACAACAATCCGATTATGTATTAAATCCTACACAAGCACACA  2800
GCTGATGTAAGCAAATGTAATGTACAATATTTGTCATTAAAAAACAGAAAGGCTTATTAATGTAATTATTATTGTATTTAATTACCAAACGAAAACGAA  2900
TAAACATTCGAGTCATTAAGCAAACAAAC  2930
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(B)

CENTROMERE   3L   TELOMERE

10 kb

Su(H) | Kr | knl | ftz-f1 | ftz | cad | 149 122 227 192

cad | ftz | Su(H) | 215 203 | 1431

391

[10231]   [750]   [89]   [72]   [1447]   [69]   [1334]

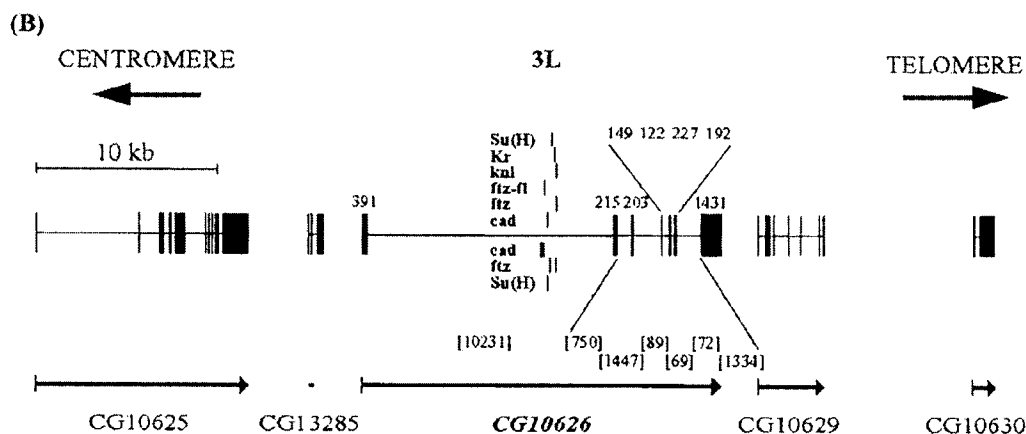CG10625   CG13285   *CG10626*   CG10629   CG10630

FIG. 3. **CG10626 gene structure.** *A*, cDNA and putative protein sequence of *CG10626*. Downstream promoter element promoter consensus sequences at +24 and +28–33 are marked in *bold* and highlighted. The polyadenylation signal and start and stop codons are also in *bold* and highlighted. Predicted transmembrane domains are *underlined*, while the peptide sequence used to make an antiserum is indicated by a *shaded box*. Potential *N*-glycosylation sites (‡) and the positions of introns (><) are marked. *B*, genomic context of *CG10626*. View of region 64E of
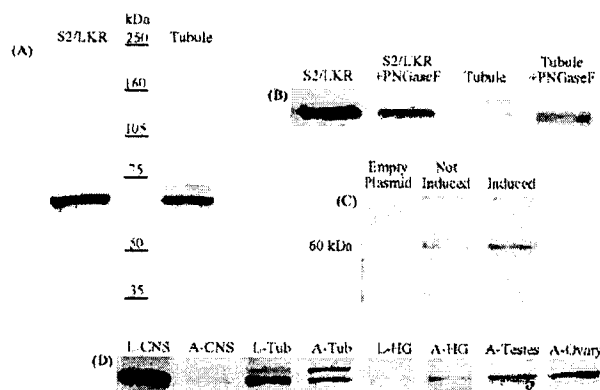
FIG. 4. **Western blot analysis of the DLKR protein.** *A*, anti-DLKR antiserum is specific. 15 μg of protein from S2 cells (*left*) and tubule (*right*) expressing DLKR was run on SDS-PAGE and analyzed by Western blot using enhanced chemiluminescence. *B*, DLKR is *N*-glycosylated. S2/LKR (*left pair*) and tubule protein (*right pair*) samples were treated with peptide:*N*-glycosidase F to remove *N*-linked carbohydrate side chains. 15 μg of each sample was analyzed by Western blot using anti-LKR peptide antibody. *C*, expression of DLKR in *E. coli*. *E. coli* were transformed with empty vector, or a vector containing the DLKR ORF, and expression induced. The induced band was of the size predicted from the cDNA (60 kDa). *D*, tissue-specific expression of DLKR. Tissue samples analyzed are larval central nervous system (*L-CNS*), adult central nervous system (*A-CNS*), larval tubule (*L-Tub*), adult tubule (*A-Tub*), larval hindgut (*L-HG*), adult hindgut (*A-HG*), adult testes (*A-Testes*), and adult ovary (*A-Ovary*). 15 μg of each protein sample was analyzed using anti-LKR peptide antibody.

is used. A polyadenylation signal is also present within the 3'-untranslated region of the sequence (AATAAA, 26 bp from poly-adenylation site). The full transcript contained within RE03009 spans two genomic scaffolds, AE003565 and AE003566, a total of 16.9 kb containing 8 exons in all. No P-element insertion lines are available in the region of this gene. There is no TATA box consensus, but there is a perfect downstream promoter element promoter consensus sequence at sites +24 (G) and +28–33 (GGTTGT). The majority of this genomic sequence (Fig. 3*B*) is comprised of a 10.7-kb intron between exons 1 and 2, which is prior to the ATG start codon so is suggestive of the presence of enhancer sequences within this large intron. In fact, using the cis-analyst tool there is a tight cluster of seven *Drosophila* transcription factor binding sites predicted within this intron (Fig. 3*B*).

The DLKR Protein Is N-Glycosylated and Found in Multiple Tissues—Western blot analysis using an anti-CG10626 peptide raised antibody identified a single major band in both *Drosophila* Malpighian tubules and DLKR-expressing S2 cells (Fig. 4*A*). This band was heavier than the predicted size of 60.6 kDa for DLKR protein, migrating at about 70 kDa, and the tubule band was consistently a doublet, implying that the receptor is modified more extensively in this tissue than in cell lines. This is indicative of post-translational modifications, such as glycosylation or myristoylation. There are three potential *N*-glycosylation sites within the protein sequence of CG10626, at Asn[183], Asn[187], and Asn[197], within the putative second extracellular loop, all with the consensus sequence N-*X*-S/T (Fig. 3*A*). This is consistent with the sites of glycosylation observed in the immature *Drosophila* rhodopsin protein (33). Glycosylation is known to occur to several neuropeptide receptors and
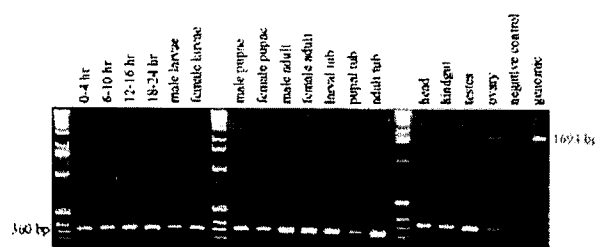

FIG. 5. **RT-PCR expression profiling of *CG10626*.** RT-PCR was performed on staged embryo; male and female larvae, pupae, and adult; larval, pupal, and adult tubules; head, hindgut, testes, and ovary cDNA; and genomic DNA. The negative control contained primers, but no template. The expected fragment sizes for cDNA and genomic are 360 and 1693 bp, respectively, sized using a 1-kb DNA ladder (Invitrogen); accordingly, authentic cDNA could be distinguished from genomic DNA by product size.

can significantly increase the molecular weight of a native protein (up to or above 5 kDa per site) by the addition of carbohydrate side chains. The majority of glycosylation is via *N*-linking to Asn, and this is also the type of glycosylation that contributes the majority of any weight increase. Accordingly, treatment of tubule protein with peptide:*N*-glycosidase F, an enzyme that removes *N*-linked carbohydrate chains, revealed a lower molecular weight band by Western blot analysis (Fig. 4*B*). In *E. coli*, where expressed proteins are not glycosylated, the cDNA gave a product of the calculated size of 60 kDa (Fig. 4*C*), implying that eukaryotic-specific post-translational modifications were responsible for the higher molecular masses seen in *Drosophila* cells and tissues (Fig. 4*A*).

Analysis of the levels of the LKR protein in different tissues reveals that there is far greater expression in larval brain than adult brain (Fig. 4*D*). Likewise, there is slightly higher expression in the larval tubule than adult, although the converse is true for hindgut. Unexpectedly, there is also significant expression in adult testes and ovary.

Intron-spanning primers were designed to the *CG10626* sequence and used for RT-PCR analysis of various tissues and developmental stages (Fig. 5). A distinct fragment of the correct size (360 bp) was detected in each sample. This suggests that this gene is widely expressed throughout all developmental stages, in both male and female, and in multiple tissues, all consistent with the Western blot analysis.

Immunocytochemistry with anti-DLKR antibody shows that, within the Malpighian tubules, the protein is only present in the secondary stellate cells and appears to be concentrated to the basolateral membrane, as would be expected for a hormone receptor (Fig. 6, *A* and *B*). The protein is also present in larval and adult central nervous system. In larval central nervous system, cell bodies stain brightly in the *pars intercerebralis* between the two lobes of the brain; there is also extensive staining in the neuropil (Fig. 6, *C* and *D*). Extensive dendritic fields are labeled as well as somata. Staining in the adult central nervous system is more restricted, only brightly staining cell bodies in the *pars intercerebralis* of the adult brain (Fig. 6*E*). This is in an area of the brain where the release of neurohormones is known to occur (34). Previous data have emphasized the role of Drosokinin as a hormone acting in peripheral tissue; these results show that there are also multiple targets throughout the central nervous system.

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chromosome 3L. The neighbors of *CG10626* are *CG13285* upstream and *CG10629* downstream; all these predicted genes are of unknown function. All genes in this region are on the negative strand. The genomic sequence for *CG10626* is contained in the genomic scaffold (GenBank™ accession numbers AE003565 and AE003566). Exons and introns are drawn to scale, lengths are marked in bp, and intron sizes are *bracketed*. The transcription factor binding site cluster is predicted by the cis-analyst tool (29). Binding sites predicted are for suppressor of Hairless (*Su(H)*), kruppel (*Kr*), knirps (*kni*), ftz transcription factor 1 (*ftz-f1*), fushi tarazu (*ftz*), and caudal (*cad*). There are no other similar clusters within this region.
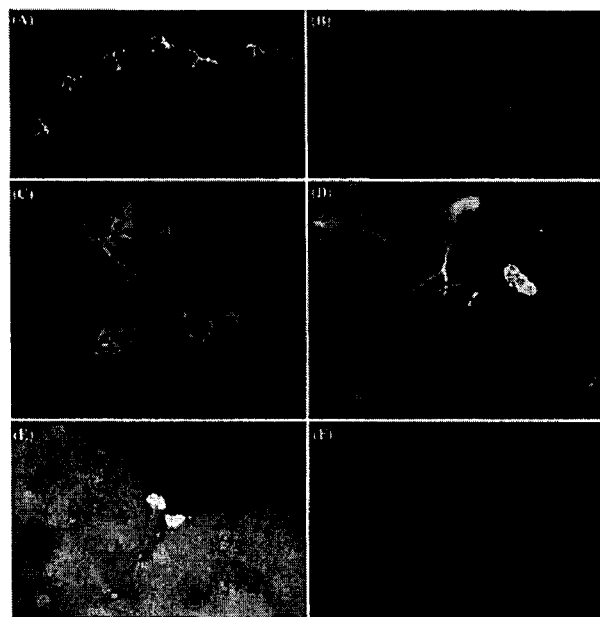
FIG. 6. **Immunocytochemical localization of the DLKR protein.** Tissues were stained with rabbit polyclonal anti-DLKR peptide antibody, raised as described in the text. Fluorescein or Texas Red secondary antibodies were used to visualize staining. The epitope (GIYNGSS-GQNNNVN) is located in the intracellular C-terminal domain of the predicted protein sequence. *A*, adult Malpighian tubule. Fluorescence microscopy of immunostained whole mount tubules revealed staining only in the secondary stellate cell type, concentrated to the basolateral membrane. No staining was seen in the principal cell type (for scale, tubule diameter is ~35 μm). Magnification: ×200. *B*, Adult Malpighian tubule-preimmune serum. Tubule was processed and exposed as for *A*, but with preimmune serum, confirming the specificity of the antibody. *C*, larval central nervous system. Fluorescence microscopy of immunostained whole larval central nervous system revealed staining in specific cell bodies within the *pars intercerebralis* as well as extensive staining in neuropil, dendritic fields, and somata. Magnification: ×200). *D*, larval central nervous system at different focal plane. Prominent groups of cells in the *pars intercerebralis* are evident. Magnification: ×500. *E*, adult central nervous system. Fluorescence microscopy of immunostained whole adult central nervus system revealed more limited staining, confined to six brightly labeled cell bodies within the *pars intercerebralis*. Magnification: ×200. *F*, adult central nervous system-preimmune serum. Central nervous system was prepared and stained as for *C* and *D*, except that preimmune serum was used, confirming the specificity of the antibody.

Novel Functional Roles for DLKR—It has already been shown that Drosokinin acts through intracellular calcium in the Malpighian tubule (7, 20). However, our results showed DLKR to be widely expressed, both in known (hindgut), plausible (brain), and unknown (testes, ovary) targets of leucokinin signaling. It was thus important to establish whether there was a functional correlate of this expression. Measurements of intracellular calcium in whole tissues confirmed DLKR protein was functional in larval central nervous system, and in adult hindgut, testes, and ovary (Fig. 7). In all cases, there is a biphasic response, with a prolonged second phase; in some tissues, the initial rapid response is almost undetectable. These increases are significant, but can only be taken as quantitative in tubules, where a GAL4 driver is available with a perfect match to the expression pattern of DLKR. In brain hindgut, testes, and ovary, the heat-shock promoter causes ubiquitous expression of the aequorin reporter, but the fraction of cells responding to Drosokinin is unknown. Nonetheless, this effect could only cause an underestimation of the calcium response and does not compromise the significance of the result.
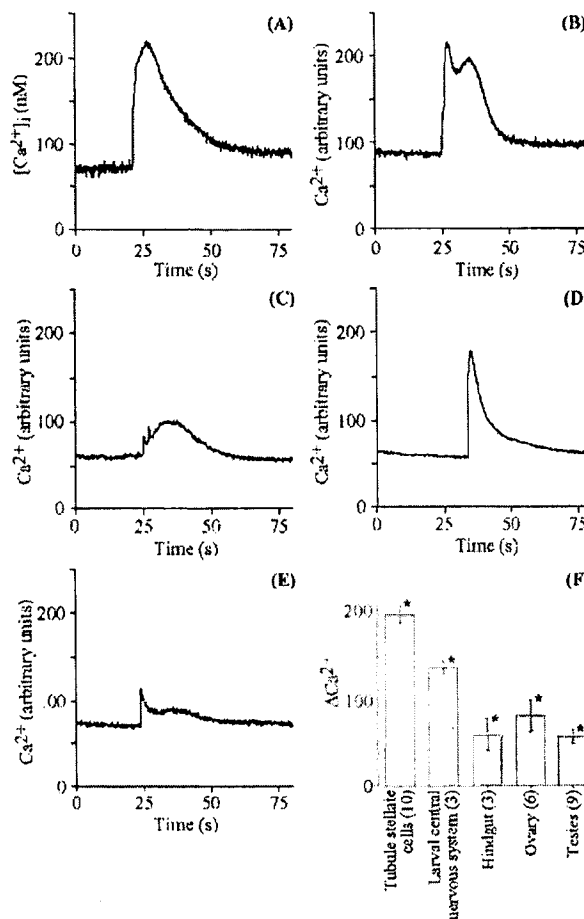

FIG. 7. **Real-time measurements of intracellular calcium in intact tissues.** Traces of $[Ca^{2+}]_i$ in intact tissue when stimulated with 10^{-7} M Drosokinin. Data are expressed as $[Ca^{2+}]_i$ (nM) against time (s); each data point corresponds to 0.1 s. *A*, adult tubule; *B*, larval central nervous system; *C*, adult hindgut; *D*, adult ovary; *E*, Adult testes. *F*, summary of calcium changes induced by Drosokinin in target tissues. Values are means of calcium increase over basal values, with the number of repetitions in *brackets*; significant changes are denoted by an *asterisk*. As explained in the text, the values for tissues other than tubule are approximate.

DISCUSSION

The leucokinin family of insect diuretic peptides has attracted great interest, both from a basic scientific viewpoint, and as possible lead compounds for insecticide development. However, although a plausible insect candidate had been identified (18, 19), this was based on an asserted distant similarity of the snail neuropeptide (which did not perfectly match the canonical leucokinin SWGamide C-terminal), to the insect peptide family (Fig. 1), and there was no supporting functional evidence for this assignment. Here, we have shown that of the GPCRs that were closely similar both to lymnokinin and other tachykinins, only a single gene within the whole *Drosophila* genome, *CG10626*, really is a leucokinin receptor. The functional properties of the receptor, when expressed heterologously in cell lines, are so similar to those we have previously inferred from physiological studies of leucokinin action on the Malpighian tubule that we are confident that we have obtained the cognate receptor. This does not, of course, exclude the possibility that further leucokinin receptors are among the other 100 or so GPCRs in the *Drosophila* genome. However, multiple receptors for given ligands, even where they act through different

second messengers, tend to co-segregate within the dendrogram (*e.g.* serotonin receptors; Fig. 1), so we think that the possibility of there being other receptors in this species is remote.

Now that the functional similarity between leucokinin-like receptors in snail, tick, and insect has been established, we can also infer that leucokinin signaling is phylogenetically widely distributed among invertebrate phyla.

Leucokinins were originally characterized as affecting hindgut motility. Consistent with this, the receptor is expressed in the hindgut. Stimulation of circular muscles in the hindgut would lead to an increase in peristalsis, as originally reported in *L. maderae* hindgut (2). However, in this paper, we have identified two areas of gene expression, by RT-PCR or immunocytochemistry, that had not previously been expected. The first is an extensive staining of the central nervous system, particularly in areas associated with neuropeptide secretion, and the second is in both male and female genital tracts. Expression in both of these domains is functional, as we were able to show significant stimulation of calcium upon leucokinin application. This is thus the first demonstration that leucokinins act on the insect genital tract and may be of significance in fertility or in the peristaltic transfer of sperm or eggs.

However, the tissue in which leucokinin signaling has been characterized in most detail is in the Malpighian tubule. In *Drosophila*, the EC_{50} for stimulation of fluid production has previously been shown to match that for elevation of intracellular calcium, and leucokinin was additionally shown not to raise tubule cAMP or cGMP, allowing calcium to be identified as the second messenger (7). Here, we showed that the EC_{50} for the heterologously expressed receptor is exactly concordant with these previously determined values. We are thus confident that the action of Drosokinin on Malpighian tubules can be explained by its interaction with the receptor we have characterized here.

Leucokinins are known to stimulate fluid production by increasing the chloride shunt conductance through the epithelium. Other workers have ascribed this to paracellular pathways in other Diptera (39). However, we have shown the presence of high conductance chloride channels in tubules and have demonstrated that chloride current hotspots invariably map to stellate cells (16). Additionally, we used tubules in which aequorin had been targeted specifically to either principal cells or stellate cells to show that only the latter responded to calcium by an increase in intracellular calcium (7). Consistent with this, our data show that the Drosokinin receptor is expressed only in stellate cells. We can thus now be very confident that, in *Drosophila* at least, leucokinin acts by raising calcium in only stellate cells and that this is sufficient to activate chloride shunt conductance. Now that a prototypic insect leucokinin receptor has been identified and characterized in detail, the scope of this model in other Diptera and other insect orders should be easy to establish.

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ISOLATION AND CHARACTERIZATION OF A LEUCOKININ-LIKE PEPTIDE OF DROSOPHILA MELANOGASTER

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Summary

The leucokinin (LK) family of neuropeptides has been found widely amongst invertebrates. A member of this family was purified from adults of the fruit fly Drosophila melanogaster. The peptide sequence for Drosophila leucokinin (DLK) was determined as Asn-Ser-Val-Val-Leu-Gly-Lys-Lys-Gln-Arg-Phe-His-Ser-Trp-Gly-amide, making it the longest member of the family characterized to date. Synthetic DLK peptide was shown to act to stimulate fluid secretion in D. melanogaster Malpighian (renal) tubules by approximately threefold, with an EC_{50} of approximately 10^{-10} mol l^{-1}, and a secondary effect at approximately 10^{-7} mol l^{-1}. DLK also acted to elevate intracellular [Ca^{2+}] in the Malpighian tubules by approximately threefold, with an EC_{50} of 10^{-10} to 10^{-9} mol l^{-1}. Responses were detected in stellate cells and occasionally in principal cells, although at no concentration tested did [Ca^{2+}] in the principal cell increase significantly above background. In stellate cells, DLK produced a biphasic rise in intracellular [Ca^{2+}] from resting levels of 80–100 nmol l^{-1}, with a transient peak being followed by a slower rise that peaked at 200–300 nmol l^{-1} after 3 s, then decayed over approximately 10 s. The wide range of concentrations over which DLK acts suggests the involvement of more than one receptor. The genomic sequence encoding the DLK peptide has been identified, and the gene has been named pp. The gene resides at cytological location 70E3–70F4 of chromosome 3L. The localisation of this first Drosophila LK gene in a genetic model permits a genetic analysis of the locus.

Key words: Drosophila melanogaster, neuropeptide, leucokinin, Malpighian tubule, aequorin, calcium.

Introduction

The integration of extracellular signals to produce an appropriate cellular response is central to our understanding of organismal function. In the analysis of such systems, the use of genetic manipulation can potentially prove invaluable, as it allows the effects of mutagenesis of specific components of the pathway to be studied in an otherwise normal organism. For such studies, the fruit fly Drosophila melanogaster provides probably the best balance between genetic power and experimental tractability (Rubin, 1988).

At present, the best D. melanogaster model system for the analysis of neurohormonal control is provided by the renal, or Malpighian, tubules (Dow et al., 1994b, 1998), which regulate salt and water balance by transepithelial secretion. These four simple epithelial tubular structures are composed of precisely determined numbers of cells of multiple types that can be genetically tagged by transposon-based enhancer trapping (Sözen et al., 1997). The main segment of each tubule (Fig. 1) is the chief site of fluid secretion and electrolyte regulation, and it is composed of interspersed Type I (principal) and Type II (stellate and bar-shaped) cells (Sözen et al., 1997).

Fluid production by the main segment of the tissue is energized by a vacuolar H$^+$-motive ATPase, located in the apical membrane of the large principal cells, where it drives a net secretion of K$^+$ via an amiloride-sensitive K$^+$/H$^+$ exchanger (Davies et al., 1996; Dow et al., 1994b, 1998; O'Donnell et al., 1996; Sözen et al., 1997). Chloride flows through maxi-Cl$^-$ channels in the stellate cells to balance the charge transfer (O'Donnell et al., 1996, 1998), and water is thought to flow transcellularly via aquaporins in the stellate cells (Dow et al., 1995). A range of organic solutes are actively transported by the main segment principal cells (Sözen et al., 1997).

Our detailed understanding of these transport processes is balanced by a knowledge of the signalling pathways that control them (Fig. 1). The V-ATPase is stimulated by a rise in either cyclic AMP or cyclic GMP level (Dow et al., 1994a,b; O'Donnell et al., 1996); although the extracellular ligand for the former is unknown, the cardiac acceleratory peptide CAP$_{2b}$ is known to act through intracellular Ca^{2+} in principal cells to stimulate an intrinsic nitric oxide synthase (DNOS) to produce NO and so raise cyclic GMP concentration through the action
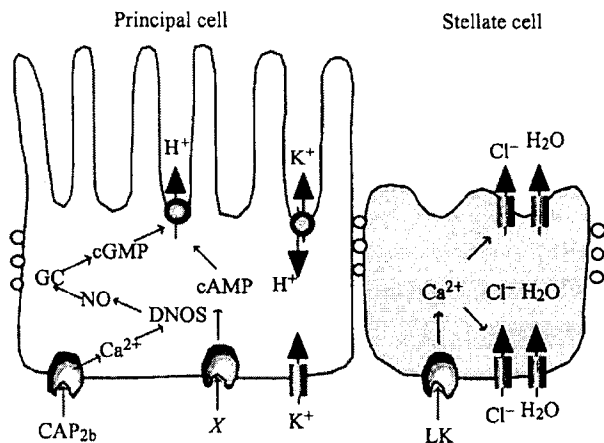
Fig. 1. Summary of the transport physiology of the main segment of the *Drosophila melanogaster* Malpighian tubule. CAP$_{2b}$, cardiac acceleratory peptide 2b; GC, guanylate cyclase; LK, leucokinin; DNOS, nitric oxide synthase, X, extracellular ligand for cyclic AMP pathway. See text for details.

of NO on a soluble guanylate cyclase (GC; Davies et al., 1995, 1997; Rosay et al., 1997).

Invertebrate leucokinins (LKs) constitute a family of myotropic neuropeptides that are active in all insect species so far studied (Cantera et al., 1992; Chen et al., 1994; Hayes et al., 1994; Holman et al., 1986a,b, 1987a,b; Nässel and Lundquist, 1991; Nässel et al., 1992; O'Donnell et al., 1996; Pannabecker et al., 1993; Schoofs et al., 1992; Veenstra, 1994). Related peptides have been identified in snails (Cox et al., 1997) and shrimp (Nieto et al., 1998), and leucokinin-related immunoreactivity has been described in a nematode (Smart et al., 1993) and an arachnid central nervous system (Schmid and Becherer, 1996). The biological activity of these peptides appears to hinge upon the presence of a C-terminal pentapeptide (Nachman et al., 1995), a feature shared with the vertebrate peptide family of tachykinins. First isolated from *Leucophaea maderae* (cockroach) on the basis of their ability to induce hindgut contractions, LK peptides can also stimulate ion transport and fluid secretion in insect renal tubules (Coast et al., 1990; Hayes et al., 1989; O'Donnell et al., 1996; Veenstra et al., 1997b), often in a cross-specific manner (Hayes et al., 1989; O'Donnell et al., 1996).

Peptides of the LK family have been shown to raise the Cl⁻ shunt conductance in insect renal tubules (Hayes et al., 1989; O'Donnell et al., 1996, 1998; Pannabecker et al., 1993). In *D. melanogaster*, it has further been shown that LKs act on stellate cells to raise intracellular [Ca²⁺], and thence to raise Cl⁻ flux specifically through stellate cells (O'Donnell et al., 1998; Rosay et al., 1997). This, in turn, provides a model, applicable at least to other Diptera, in which stellate cells are conspicuous and possibly to other insects in which such functional specialization may not be apparent (Dow et al., 1998).

Previous studies on the renal response of *D. melanogaster* to LKs have employed synthetic peptides derived from other

orders of insect (Davies et al., 1995; O'Donnell et al., 1996, 1998; Rosay et al., 1997). However, in this study, we describe the isolation and characterization of the first *D. melanogaster* member of this family, *Drosophila* leucokinin (DLK). The action of this peptide on tubules is similar to those of non-native peptides in that it raises both fluid secretion rates and intracellular [Ca²⁺]. Interestingly, the two responses occur over distinct concentration ranges, suggesting the possibility of multiple effects.

Materials and methods

Flies

Drosophila melanogaster (strain Oregon R) were raised in tubes or bottles on synthetic diet under standard conditions, as described previously (Dow et al., 1994b). Flies transgenic for the aequorin transgene, under control of the UAS promoter (UASaeq) enhancer trap lines that direct GAL4 expression to principal cells (c42), secondary cells (c710) and lower tubules (c507) and a heat-shock/GAL4 construct (hsGAL4) were those described previously (Rosay et al., 1997; Sözen et al., 1997). To produce flies with cell-specific aequorin expression, homozygous UASaeq flies were crossed to flies homozygous for any of the driver insertion lines described, and the adult progeny were used as described below (see Fig. 3).

Peptide purification

Throughout the purification procedure, leucokinin-immunoreactivity was identified by competitive enzyme-linked immunosorbent assay (ELISA), the general procedure for which followed that described elsewhere (Veenstra and Lambrou, 1995), and used an antibody prepared against leucokinin I (Nässel and Lundquist, 1991) and a leucokinin II–glutaraldehyde–thyroglobulin conjugate. This ELISA is more sensitive and less specific for a single leucokinin than the leucokinin IV ELISA used for the isolation of the mosquito leucokinins (Veenstra, 1994).

The *Drosophila* leucokinin was isolated from 400 g of the Canton S strain (adults 7–14 days old) following essentially the protocol used for the isolation and identification of 13 neuropeptides from the mosquito *Aedes aegypti* (Veenstra, 1994, 1998; Veenstra et al., 1997a), but with improvements suggested by previous results (Fig. 2). Samples of 20 g of frozen flies were homogenized in 200 ml of Bennett's mixture [5 % formic acid, 1 % trifluoroacetic acid (TFA), 1 % NaCl and 1 mol l⁻¹ HCl in water; Bennett et al., 1981] in a Waring mixer for 5 min at high speed. Extracts were centrifuged for 20 min at 13 000 g at 4 °C, and the were pellets re-extracted once. The supernatants were loaded on previously activated and equilibrated home-made 'Super-Sep-Paks', containing 7 g of preparatory C₁₈ reversed-phase beads (55–105 μm, Millipore Corporation, France), and the was material eluted, collected, lyophilized and stored as described in detail for the mosquito leucokinins (Veenstra, 1994). Preliminary experiments showed that, while some *D. melanogaster* pigments were poorly retained on such columns, no peptides of interest were lost in this step. After the entire

400 g had been processed, the combined lyophilized material was dissolved in 0.1 % of TFA, reprocessed on a single 'Super-Sep-Pak' and the peptide material lyophilized.

Four different high-performance liquid chromatography

A

400 g of *Drosophila*
Bennett's mixture

↓

Solid-phase extraction Waters C_{18}
(12.5 nm pores, 55–105 μm particles)
0–65% CH_3CN/TFA

↓

Econosil C_{18} 250 mm × 22.5 mm, 10 μm
$H_2O–CH_3CN/HFBA$

↓

Ultremex C_1 250 mm × 4.6 mm, 5 μm
$H_2O–CH_3CN/TFA$

↓

Microsorb Phenyl 250 mm × 4.6 mm, 5 μm
$H_2O–CH_3CN/TFA$

↓

Protein Pak I-125 300 mm × 7.8 mm
$CH_3CN–H_2O/TFA$

(HPLC) steps were used to purify the *Drosophila* leucokinin. The first HPLC step used an Econosil C_{18} reversed-phase column (250 mm×22.5 mm, Altech Associates Inc., Deerfield, IL, USA). The column was equilibrated in 0.1 % heptafluorobutyric acid (HFBA) in water at a flow rate of 10 ml min^{-1}. After injection of the final lyophilisate redissolved in 0.1 % HFBA, the column was run isocratically for 10 min, after which a linear gradient to 16 % CH_3CN and 0.1 % HFBA in water over 20 min was started, followed immediately by a linear gradient over 90 min to 52 % CH_3CN and 0.1 % HFBA in water. Fractions were collected every 48 s and analysed by ELISA for the presence of leucokinin-immunoreactive peptides. Absorbance was measured at 280 nm.

Fractions containing leucokinin-immunoreactivity were identified and further purified on an Ultremex C_1 reversed-phase column (250 mm×4.6 mm; Phenomenex, Torrance, CA, USA). This column was equilibrated with 0.1 % TFA in water and eluted at 1 ml min^{-1}. After injection of the leucokinin-immunoreactive material, the column was eluted isocratically with 0.1 % TFA for 10 min, followed by a linear gradient over 60 min to 13 % CH_3CN and 0.1 % TFA in water, and a second linear gradient over 30 min to 32.5 % CH_3CN and 0.1 % TFA in water. Fractions were collected every minute and analysed for leucokinin immunoreactivity. Absorbance was measured at 214 and 280 nm.

The third HPLC purification step used a Microsorb Phenyl reversed-phase column (250 mm×4.6 mm; Rainin Instrument Company, Inc., Woburn, MA, USA). This column was equilibrated with 13 % CH_3CN and 0.1 % TFA in water; after injection of the leucokinin-immunoreactive material, a linear
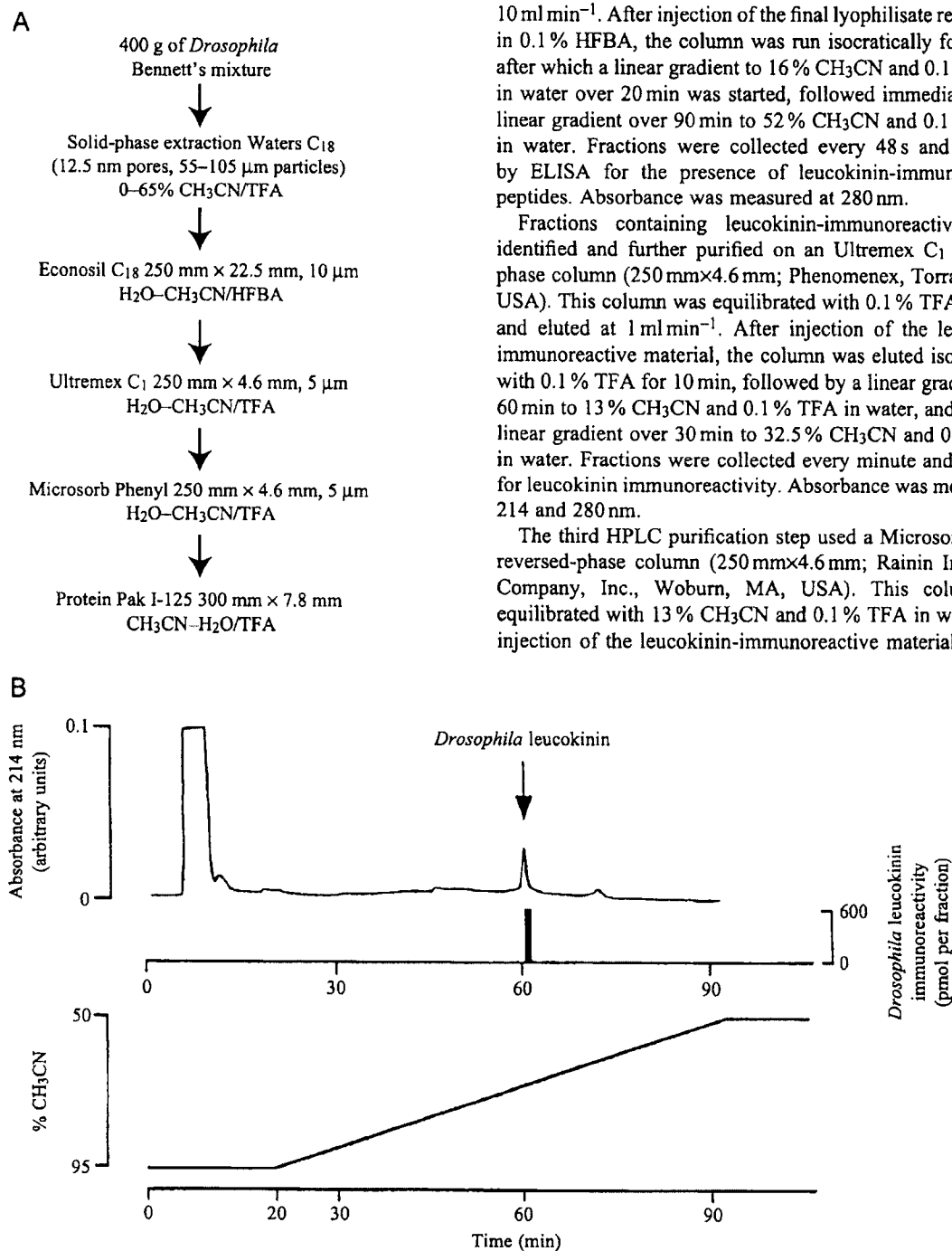
B

Fig. 2. Procedure for the isolation of *Drosophila* leucokinin (DLK). (A) Flow chart of isolation procedure. (B) HPLC elution profile of the crude extract. The peak that was collected is marked with an arrow. Abbreviations for chemicals are explained in Materials and methods.

gradient over 10 min to 19.5 % CH3CN and 0.1 % TFA in water was started, followed immediately by a second linear gradient over 60 min to 32.5 % CH3CN and 0.1 % TFA in water, and finally a gradient over 15 min to 45.5 % CH3CN and 0.1 % TFA in water. Fractions were collected at 1 min intervals and analysed by ELISA for leucokinin immunoreactivity. Absorbance was measured at 214 and 280 nm.

The final HPLC step was performed on a Protein Pak I-125 column (300 mm×7.8 mm; Millipore Corporation), used in normal phase mode. The column was equilibrated in 95 % CH3CN and 0.01 % TFA in water at a flow rate of 1.5 ml min^{-1}. After injection of the leucokinin-immunoreactive material, the column was eluted isocratically for 10 min, followed by a gradient over 80 min to 50 % CH3CN and 0.01 % TFA in water. Fractions were collected every 2 min and absorbance was measured at 214 and 280 nm.

The isolated peptide was sequenced on an automated protein sequencer (model 494A, Perkin Elmer Applied Biosystems Procise) by the Unité de Recherche de Biochimie et Structure des Protéines of INRA at Jouy-en-Josas. *Drosophila* leucokinin was synthesized by the Unité de Biophysique Structurale, Université Bordeaux I on an automated peptide synthesizer (model 341A, Applied Biosystems).

Fluid secretion assay

Fluid secretion experiments were performed on 3- to 7-day-old adults of *D. melanogaster* as described previously (Dow et al., 1994b). Tubule pairs were placed in 10 μl drops of Schneider's medium under paraffin oil, and one tubule of the pair was anchored to a metal pin outside the drop. Fluid produced by the remaining tubule generated a small droplet at the urethra under oil. These were collected at 10 min intervals, the diameter measured and their volumes calculated. DLK was serially diluted in Schneider's medium and added to sets of (usually 10) tubules immediately after the 30 min reading, and fluid production was measured for a further 30 min.

Aequorin measurement of intracellular [Ca^{2+}]

The effects of DLK and other peptides on intracellular [Ca^{2+}] in principal and stellate cells were measured as described previously (Fig. 3) (O'Donnell et al., 1998; Rosay et al., 1997). Briefly, tubules were dissected from adult (3–10 days old) flies, pooled in groups of 20, and incubated in Schneider's medium containing 2.5 μmol l^{-1} coelenterazine for 2–4 h. Luminescence recordings were made with a Berthold–Wallac luminometer. Tubule samples (one per data point) were mock-injected with Schneider's medium, followed by application of test solutions in Schneider's medium. Responses were measured over a period of 1 min. At the end of each experiment, the total luminescence of each tubule sample was assessed by injecting 100 mmol l^{-1} Ca^{2+}/1 % Triton X-100 to permeabilise the cells and so discharge the remaining aequorin. This allowed luminescence rates to be converted to Ca^{2+} concentration by reverse integration with a program written in Perl, as described previously (Rosay et al., 1997).

Cyclic nucleotide assays

Intracellular cyclic AMP and cyclic GMP concentrations were measured by radioimmunoassay (Amersham Biotrak Amerlex M kits) as described previously (Davies et al., 1995). Briefly, tubules were dissected in Schneider's medium and exposed to the desired concentration of DLK for 10 min in the presence of the non-specific phosphodiesterase inhibitor, isobutylmethyl xanthine (IBMX), before lysis and assay by competitive immunoassay according to the manufacturer's instructions.

Identification of the gene encoding DLK

Because the DLK peptide sequence was too short to permit authoritative database searching, a putative propeptide sequence was constructed by adding a second glycine at the C terminus, to act as an amidation signal, and flanking the resulting sequence with lysine-arginine dibasic cleavage signals. The resulting sequence was used for low-stringency TBLASTN searches of the Genbank and Berkeley Drosophila Genome Project (BDGP).

Expression of the putative gene, identified by the searches, was verified by reverse-transcription polymerase chain reaction (RT–PCR) using primers that matched the genomic
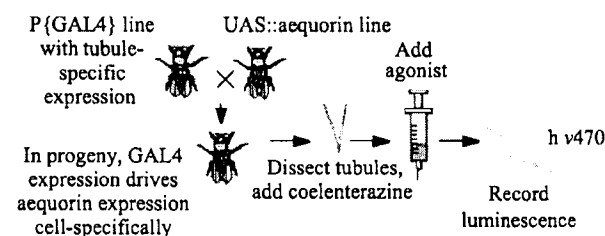

Fig. 3. Measurement of intracellular [Ca^{2+}] by cell-specific expression of aequorin. The P{GAL4} transposon is capable of driving expression of any transgene that has a UAS promoter in a pattern that reflects the genomic context of the P{GAL4} insertion. A collection of P{GAL4} lines that drive expression in particular regions or cell types of Malpighian tubules has been characterised previously. In this experiment, flies from line c42 (homozygous for a P{GAL4} insertion that can drive transgene expression in principal cells of the main segment of the Malpighian tubule) or from line c710 (homozygous for a P{GAL4} insertion that can drive transgene expression in stellate cells) were mated to flies homozygous for a second chromosome insertion of the *Aequorea victoria* apoaequorin gene under control of the UAS promoter (Rosay et al., 1997). In the progeny of such flies, apoaequorin was expressed in the appropriate cell-specific pattern (Rosay et al., 1997). Tubules were dissected from 3- to 10-day-old adults and incubated with coelenterazine to permit the reconstitution of active aequorin. Ca^{2+} levels were measured in a luminometer with two automated injection channels, allowing real-time monitoring of light emission at 470 nm (h v470) in response to *Drosophila* leucokinin (DLK) application. At the end of the experiment, the remaining aequorin (>98 %) was discharged by permeabilisation of the tubules with Triton X-100, allowing the conversion of relative light intensity to real-time [Ca^{2+}] by reverse integration as described previously (Rosay et al., 1997).

fraction, and head mRNA as the template. This procedure has been described elsewhere (Davies et al., 1977).

Results

Peptide purification

After the first HPLC step, a leucokinin-immunoreactive peak was detected in fractions 113, 114 and 115, corresponding to a retention time of 89.6–92.0 min. The immunoreactive material eluted from the C_1 column with a retention time of 36–38 min, and subsequently on the phenyl column it was recovered between 31 and 33 min. The final HPLC column yielded one major peak at 63.4 min containing all leucokinin-immunoreactive material and three minor contaminants which were well separated from the *Drosophila* leucokinin. Sequence analysis yielded unambiguously the following sequence, Asn-Ser-Val-Val-Leu-Gly-Lys-Lys-Gln-Arg-Phe-His-Ser-Trp-Gly and showed that a total of approximately 600 pmol had been purified.

As all leucokinins identified so far are C-terminally amidated, and the leucokinin I ELISA used did not recognize a synthetic non-amidated analogue of *Aedes* leucokinin II and thus appears to recognize only C-terminally amidated peptides, the *Drosophila* leucokinin was synthesized with a C-terminal amide. The synthetic *Drosophila* leucokinin had the same retention time on HPLC as the isolated peptide and thus confirms the presence of the C-terminal amide in the natural peptide, since oligopeptide analogues differing only in the presence or absence of a C-terminal amide differ significantly (approximately 2 min) in retention time under the conditions used. Hence, the structure of *Drosophila* leucokinin is Asn-Ser-Val-Val-Leu-Gly-Lys-Lys-Gln-Arg-Phe-His-Ser-Trp-Gly-amide (this sequence has been deposited in the SwissProt database with the accession number P81829).

Fluid secretion assay

The DLK peptide produced a clear stimulation of fluid secretion, with a time course similar to that seen for other members of the leucokinin family (Fig. 4A). Typically, the tubule responded maximally in less than the sampling interval, and the increase in fluid secretion rate was sustained over many minutes. The extent of the increase in fluid secretion (approximately 2–3 nl min^{-1}) is comparable with that observed previously in *D. melanogaster* tubules for non-native leucokinins. The dose–response curve (Fig. 4B) shows that half-maximal effect is seen between 10^{-11} to 10^{-10} mol l^{-1}, and shows significant additional stimulation above 10^{-7} mol l^{-1}.

Aequorin measurement of intracellular [Ca^{2+}]

DLK produced a rapid elevation of intracellular [Ca^{2+}] in stellate cells (Fig. 5), as we have reported for other members of the leucokinin family. However, the authentic DLK peptide elicited a rapid transient response in the first time-point (100 ms after injection), followed by a slower rise in [Ca^{2+}] that reached a higher peak 3 s after injection, then
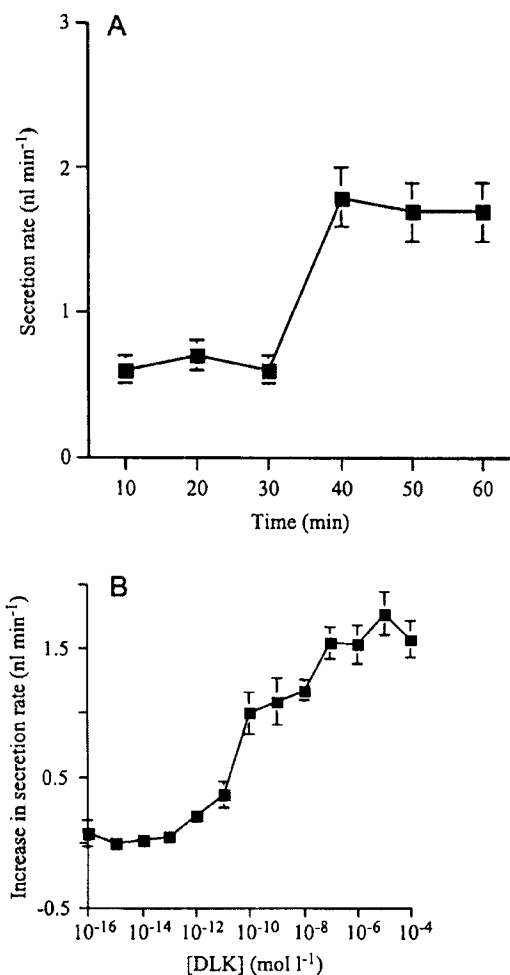

Fig. 4. Effects of *Drosophila* leucokinin (DLK) on rates of fluid secretion by Malpighian tubules. (A) Typical experiment, showing the response of tubule fluid secretion rate to DLK, added to a final concentration of 10^{-9} mol l^{-1} at 30 min. (B) Dose–response curve for the effect of DLK on fluid secretion rate. Data are presented as means ± S.E.M., $N>10$.

decayed rapidly within 10–20 s (Fig. 5). The time course of the slower peak was not sensitive to the concentration of DLK (Fig. 5). The approximately 200 nmol l^{-1} increase in cytosolic [Ca^{2+}] above a resting level of 70 nmol l^{-1} (Fig. 5) is consistent with values we have previously reported for non-native peptides (O'Donnell et al., 1998), and with the known EC$_{50}$ for *Drosophila* calmodulin, of approximately 250 nmol l^{-1}.

In contrast, DLK had no effect on intracellular [Ca^{2+}] in principal cells, as delineated by line c42 (Figs 5, 6). Principal cell responses were seen occasionally, with a similar time course to those observed in stellate cells; however, these sporadic results did not constitute a statistically significant response (Fig. 6).
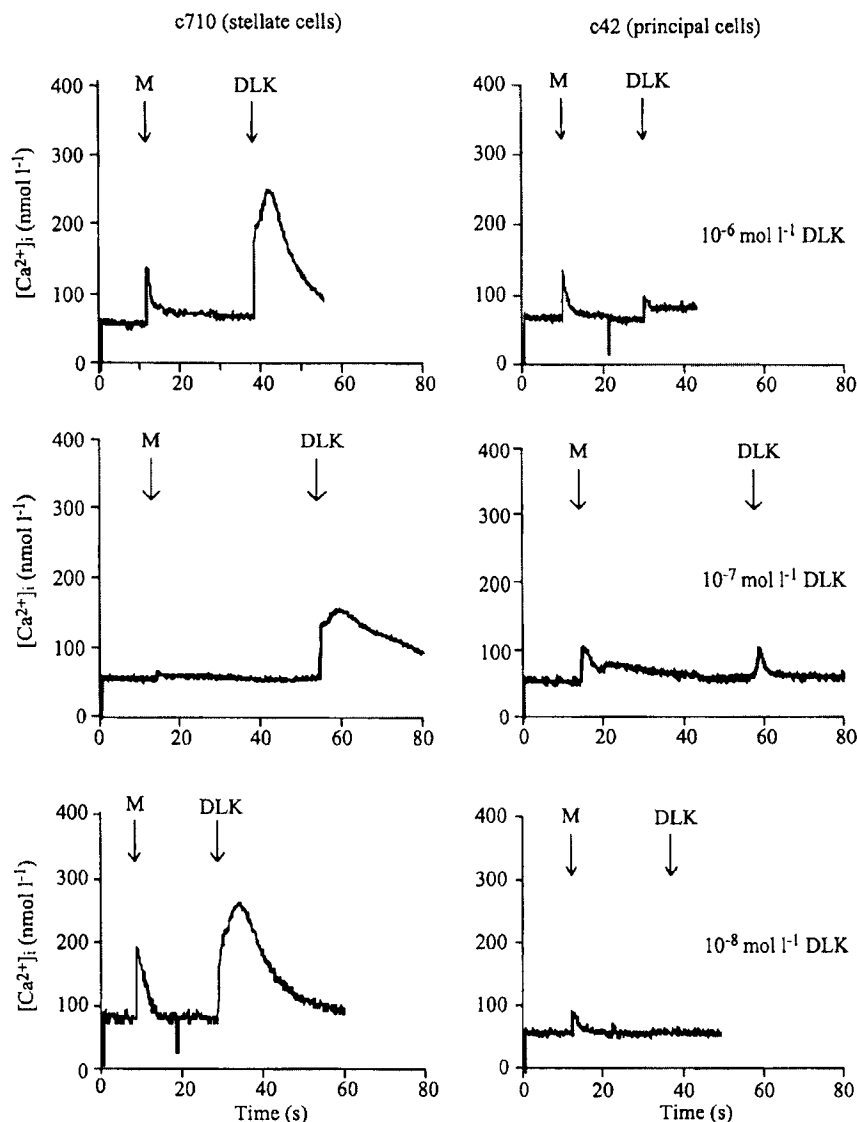
Fig. 5. *Drosophila* leucokinin (DLK) stimulates an increase in intracellular [Ca²⁺] in stellate cells. Typical intracellular $[Ca^{2+}]$ traces for stellate (left-hand panels) and principal (right-hand panels) cells, for resting cells and after mock injection of vehicle (M) or of DLK at $10^{-6}\,mol\,l^{-1}$ (top panels), $10^{-7}\,mol\,l^{-1}$ (middle panels) or $10^{-8}\,mol\,l^{-1}$ final concentration (bottom panels).

Cyclic nucleotide assays

By contrast with its effects on intracellular [Ca²⁺], DLK did not affect cyclic AMP levels in tubules (Fig. 7). This is consistent with our previous data for non-native peptides (Davies et al., 1995). DLK elicited a small reduction in cyclic GMP levels in tubules (Fig. 7). This might imply some cross-talk between the two signalling pathways or the existence of multiple DLK receptor subtypes; however, the significance of this is not clear.

Identification of the gene encoding DLK

Using the putative propeptide sequence, no hits were obtained on the 80 000 expressed sequence tags generated by the Berkeley Drosophila Genome Project, implying that DLK is a low-abundance transcript. However, a recent genomic sequence (Genbank Accession AC006496), corresponding to the first-pass sequence of Bacterial Artificial Chromosome (BAC) clone BACR48007, contains a DNA sequence that encodes the putative propeptide completely (Fig. 8). Although this preliminary sequence is too short to permit the full identification of the open reading frame (and thus of any related peptides encoded by the same gene), it is sufficient to localise the gene to 70E3–70F4 on chromosome 3L. Expression of the putative gene was verified by RT–PCR off head mRNA template. The resulting products were sequenced on both strands, and the sequence deposited in the GenBank data bank, with the accession number AF192342.

The gene encoding the DLK peptide was named *pp*. Although there are no obvious candidate loci that might

Fig. 6. Effects of *Drosophila* leucokinin (DLK) on intracellular [Ca²⁺]. Data are derived from traces for stellate cells (see left-hand panel in Fig. 5) and principal cells (see right-hand panel in Fig. 5). For each cell type and concentration, mean Ca²⁺ concentrations, throughout the experiment, are shown as initial background levels (white columns), maximum response to mock injection (light grey columns), second background reading (dark grey columns) and maximum response to DLK (black columns). A 'clean' experiment is therefore one in which the background and mock readings (the first three columns in a series) are all very similar. Data are presented as means ± s.e.m. (*N*>4); significant responses to DLK, compared with mock injection, are marked with an asterisk (Student's *t*-test for matched samples, two-tailed, taking critical level for *P*=0.05).
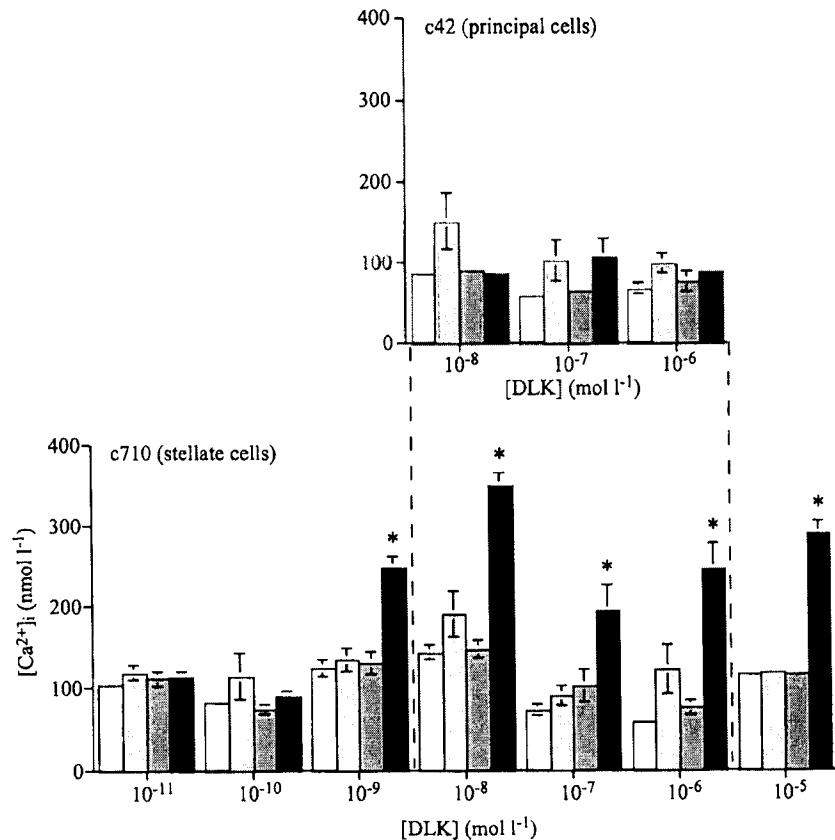
correspond to this gene, the proximity of several P-element insertion stocks means that mutagenesis of the locus should prove straightforward.

Discussion

Peptide purification

In our previous peptide isolation work on the mosquito *Aedes aegypti*, we noticed that the difference in selectivity between the C₁₈ and phenyl reversed-phase columns was very limited, although the resolving power of the first preparative HPLC column was remarkable. From this, we reasoned that

it might be possible to eliminate the HPLC step using HFBA on the phenyl column by using HFBA as the pairing ion for the preparative column and simultaneously decreasing the fraction size for this column. Because absorbance during the first HPLC purification step at 214 nm is off-scale, this had the

Fig. 7. *Drosophila* leucokinin (DLK) has a minimal effect on tubule cyclic nucleotide levels. Tubule cyclic AMP and cyclic GMP levels were measured by radioimmunoassay, as described in the text. Treatments are: 10⁻⁸, *Drosophila* leucokinin to 10⁻⁸ mol l⁻¹ final concentration; 10⁻⁷, *Drosophila* leucokinin to 10⁻⁷ mol l⁻¹ final concentration; IBMX, control with isobutylmethyl xanthine alone; CAP₂ᵦ, positive control for cyclic GMP assay, with the cardiac acceleratory peptide CAP₂ᵦ added to 10⁻⁷ mol l⁻¹ final concentration. (There is no known agonist that raises cyclic AMP levels in *Drosophila melanogaster* Malpighian tubules.) Data are presented as means ± s.e.m. (*N*=3 independent samples, each pooled from 20 tubules). Where error bars are not shown, they are too small to reproduce.

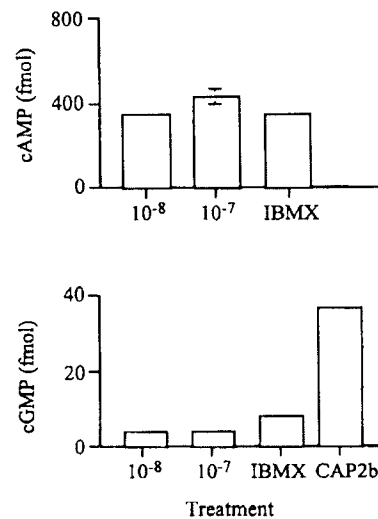Table 1. *Comparison of the* Drosophila *leucokinin (DLK) sequence with known leucokinin members*

Species	Number	Sequence	Effect on D. melanogaster tubules
Drosophila melanogaster	I	NSVVLGKKQRFHSWGamide	Yes
Aedes aegypti	I	NSKYVSKQKFYSWGamide	Yes
	II	NPFHAWGamide	No
	III	NNPNFYPWGamide	Yes
Culex salinarius		NPFHSWGamide	
Leucophaea maderae	I	DPAFNSWGamide	Yes
	II	DPGFSSWGamide	No
	III	DQAFNSWGamide	No
	IV	DASFHSWGamide	Yes
	V	GSGFSSWGamide	No
	VI	pESSFHSWGamide	Yes
	VII	DPAFSSWGamide	Yes
	VIII	GASFYSWGamide	No
Locusta migratoria	I	AFSSWGamide	
Acheta domestica	I	SGADFYPWGamide	
	II	AYFSPWGamide	
	III	ALPFSSWGamide	
	IV	NFKFNPWGamide	
	V	AFHSWGamide	
Pennaeus vannamei	I	ASFSPYGamide	
	II	DFSAWAamide	
Lymnaea stagnalis	I	PSFSSWSamide	

Residues identical to those of DLK are in bold type.

added advantage of eliminating the problems associated with working with HFBA, which is usually too impure to obtain good absorbance recordings at 214 nm. Although it is impossible to judge whether the efficacy of these improvements will be valid for other purifications from the same batch of material, we obtained excellent purification from 400 g of whole *Drosophila melanogaster* in just four HPLC purification steps.

The leucokinin peptide family

The DLK peptide is clearly a member of the invertebrate leucokinin family, sharing both the canonical -Phe-X-X-Trp-Gly-NH_2 C terminus and broader similarity with the rest of the family (Table 1). Of the known members, it is most similar in both sequence and overall length to *Aedes* leucokinin I (Veenstra et al., 1997b) although, at 15 residues, DLK is the longest leucokinin identified to date.

Tubule effects of DLK

Although originally identified in a hindgut motility assay, interest in leucokinins has focused on their diuretic actions on Malpighian tubules. In the *D. melanogaster* system, it proved possible to measure (for the first time in any insect) an increase in intracellular [Ca^{2+}] in stellate cells in response to a leucokinin (Rosay et al., 1997). Here, we have expanded these results using an authentic peptide. DLK is the most potent stimulant of fluid secretion yet identified in *D. melanogaster*; it produces a rapid, sustained increase in the rate of fluid secretion to 2–4 nl min^{-1}, with an EC$_{50}$ of less than 0.1 nmol l^{-1} (Fig. 4). This concentration makes it likely that DLK is an authentic ligand for the tubule receptor, because most neuropeptide receptors have affinities in the range 10^{-9} to 10^{-11} mol l^{-1} (Watson and Arkinstall, 1994).

The effects of DLK on intracellular [Ca^{2+}] are consistent with this model. As expected from previous studies

Fig. 8. Genomic sequence encoding *Drosophila* leucokinin (DLK). Top rows show the genomic DNA sequence corresponding to the reverse complement of bases 44013–44132 of Genbank accession number AC006496, while the lower

TCAAATCCCAGCTGCAGCGCGACGAGAAGCGCAACTCCGTCGTGCTGGGCAAGAAGCAGC
        K   **R**   N   S   V   V   L   G   **K**   **K**   Q

GATTCCACTCGTGGGGCGGCAAAAGGTCACCGGAACCACCGATCCTGCCGGACTACTAAT
R   F   H   S   W   G   *G*   **K**   **R**

rows show the corresponding amino acid sequence. Putative dibasic flanking cleavage signals are shown in bold type, while the C-terminal amidation signal is shown in italic type. Amino acids are centred on their codons.

(O'Donnell et al., 1996; Rosay et al., 1997), the primary sites of intracellular Ca^{2+} response to DLK stimulation in *D. melanogaster* tubules are stellate cells, where a rapid (100 ms) and transient Ca^{2+} 'spike' is followed by a slower Ca^{2+} 'wave' that peaks after 3 s (Fig. 5). Since increased cytosolic Ca^{2+} levels return to baseline within approximately 20 s of DLK application while the effects on fluid secretion persist for at least 30 min (Fig. 4), it is necessary to invoke a long-lived downstream mediator of Ca^{2+} signalling.

Although the fluid secretion and $[Ca^{2+}]$ data paint a persuasive picture confirming that DLK acts to stimulate fluid production through a selective action on intracellular $[Ca^{2+}]$ in the stellate cells, there is scope for further complexity. DLK elicits significant elevations of the secretion response over a very wide concentration range (10^{-12} to 10^{-4} mol l^{-1}; Fig. 4). The maximum fluid secretion rates observed at concentrations of 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} mol l^{-1} are significantly higher than those observed at 10^{-10}, 10^{-9} or 10^{-8} mol l^{-1} (Fig. 4). This bimodal response is reminiscent of the position in *Aedes aegypti* tubules, where the collapse of the apical potential (and by inference the increase in Cl$^-$ shunt conductance) occurs at a concentration higher than that required to stimulate fluid production (Veenstra et al., 1997b). Furthermore, *A. aegypti* leucokinins I and III, but not leucokinin II, have the capacity to increase the fluid secretion rate of isolated mosquito tubules (Veenstra et al., 1997b). Taken together, these lines of evidence suggest that more than one class of receptor may be involved in LK signalling in Malpighian tubules. It is possible that leucokinins modulate not only stellate cell Cl$^-$ conductance but also processes that might include the remodelling of intercellular junctions (Pannabecker et al., 1993) or the recruitment of plasma membrane water channels (Dow et al., 1995). Given the possibility that, as in *A. aegypti*, further related peptides might exist in *D. melanogaster*, it is possible that the responses elicited by DLK at high concentration are through low-affinity interaction with a receptor for a related peptide.

A common feature of vertebrate and invertebrate neuropeptide families is that individual family members tend to arise through processing of a common propeptide. For example, the three LKs identified in *A. aegypti* are encoded by a single cDNA (Veenstra, 1994; Veenstra et al., 1997b). We are accordingly investigating the gene that encodes DLK, based on the chromosomal localisation at 70E3–70F4. As well as providing information on other neuropeptides encoded by the same gene, the reverse genetic tools unique to *D. melanogaster* will provide a unique opportunity to dissect the functions of individual neuropeptides.

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